RNA SYNTHESIS DURING OOGENESIS IN XENOPUS LAEVIS

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ABSTRACT

Rates of RNA synthesis, and in particular poly(A)$^+$RNA synthesis, in oocytes of Xenopus laevis have been measured by analysing the kinetics of incorporation of exogenous radioactive nucleoside, or of microinjected radioactive nucleoside triphosphate, into TCA precipitable material coupled with determinations of precursor pool specific activity. Poly(A)$^+$RNA was isolated by oligo (dT)-cellulose chromatography.

Stable RNA, over 80% of which was rRNA, was synthesized in stage 6 oocytes and accumulated in the cytoplasm at a constant rate of 650 pg/oocyte/hour for at least 100 hours. By isolating germinal vesicles, it was shown that more than 70% of the RNA synthesized initially in stage 6 oocytes was unstable nuclear RNA with a half-life shorter than 4 hours, much of which sedimented heterogeneously on sucrose gradients. In stage 6 oocytes all the poly(A)$^+$RNA which was synthesized at an initial rate of 30 pg/oocyte/hour appeared to turn over with an average half-life of 10 hours and the steady state amount accumulated was 0.5% of the stored pool of poly(A)$^+$RNA. Similar kinetics were observed in enucleated oocytes but ethidium bromide inhibited poly(A)$^+$RNA synthesis by about 70%. These observations suggest that much of the poly(A)$^+$RNA synthesis in stage 6 Xenopus laevis oocytes is mitochondrial.

Stage 1 oocytes synthesized stable RNA at a constant rate of 17 pg/oocyte/hour for at least 80 hours and over 80% of the RNA accumulated was 4S and 5S RNA. Poly(A)$^+$RNA, with slightly different sedimentation properties and poly(A) size from that in stage 6 oocytes, was synthesized at an initial rate of 0.7 pg/oocyte/hour most of which was unstable, having a half life of about 12 hours.

The discussion relates these rate measurements to the known patterns of RNA accumulation during oogenesis and the mechanisms of transcription of maternal mRNA and other RNA classes.

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ABBREVIATIONS

Chemicals

BSA	bovine serum albumin
β-SH	2-mercapto-ethanol
CTAB	cetyl trimethyl-ammonium bromide
DEP	diethyl-pyrocarbonate
DTT	dithio-threitol
EDTA	ethylene-diamine-tetra-acetic acid
Hepes	hydroxyethyl-piperazine-ethanesulphonic acid
PAS	para-aminosalicyclic acid
PCA	perchloric acid
PPO	diphenyloxazole
PVP	polyvinyl-pyrrolidone
SDS	sodium dodecyl sulphate
TCA	trichloroacetic acid
TNS	tri-iso-propylnapthalenesulphonic acid sodium salt
Tris	tris (hydroxymethyl) aminomethane

Nucleic Acids

rRNA	ribosomal RNA
tRNA	transfer RNA (4S RNA)
hnRNA	heterogeneous nuclear RNA
mRNA	messenger RNA
poly(A)$^{+}$RNA	mRNA containing 3' poly(A)
poly(A)$^{-}$RNA	mRNA lacking 3' poly(A) or in some cases mRNA lacking sufficient 3' poly(A) to bind to oligo (dT)-cellulose
mtRNA	mitochondrial RNA
cRNA	RNA complimentary to a DNA sequence
rDNA	DNA containing genes for rRNA
mtDNA	mitochondrial DNA

CHAPTER 1

Introduction

(a) Developmental aspects of oogenesis

The amphibian ovary is developmentally established early in tadpole life (Blackler, 1966; Blackler, 1972). At the neurula stage (20 hours post fertilization) the primordial germ cells, situated in the endoderm in frogs and toads, migrate using ameoboid movement toward the dorsal midline of the embryonic posterior gut and subsequently become incorporated into the tissue of the dorsal mesentery. At about the tadpole feeding stage (100 hours) the primordial germ cells separate into equal sized right and left groups and somatic cells from the mesoderm surround and infiltrate them forming longitudinal columns on the medio-ventral face of each kidney rudiment. (Blackler, 1972; Whitington & Dixon, 1975).

Until this formation of the primary gonads, the embryonic sex cells have only divided 3 times since the blastula stage (Dziadek & Dixon, 1977), but now both these cells and the invading mesodermal cells begin to divide at an increasing rate, the gonads grow rapidly in size and towards the end of tadpole life sexual differentiation in the gonadal tissue leads to the formation of histologically distinct ovaries or testes (Blackler, 1972; Dziadek & Dixon, 1977).

During metamorphosis the two ovaries continue to grow rapidly and their 20-25 lobes increase in volume in the abdominal cavity. The lobes contain the primary oocytes which have developed exclusively from the embryonic sex cells (Witschi, 1929; Smith, 1966), the somatic mesoderm cells form the follicular tissue and the general stroma of the ovary (Blackler, 1972).

The primary oocytes of Xenopus laevis, about 10 μm in diameter begin meiosis which may take a year or more to complete and during

this time they grow in size up to 1300 μm. Dumont (1972) has prepared a useful classification for the stages of oocyte development in Xenopus laevis in which six stages are described. Table 1-1 presents the characteristics of the various oocyte stages according to Dumont (1972) and RNA contents of different oocyte stages have been added using data from Scheer (1973) and Rosbash & Ford (1974).

In immature animals up to 30 weeks post-metamorphosis the ovaries contain oocytes which are predominantly if not completely stage 1, however a few small translucent stage 2 oocytes are sometimes present especially in ovaries about 30 weeks post-metamorphosis or older.

The length of time taken for oocytes to grow to stage 6 is dependent on many factors (diet, crowding, hormones, season, environment) but it is known that the minimum time at 20°C for the production of a mature female Xenopus laevis from a fertilized egg is about one year (Ford, 1967). On the other hand Scheer (1973) showed that residual 300 μm diameter oocytes in a partially ovariectomized adult Xenopus laevis female stimulated with the hormone HCG, grew to 1100 μm in diameter in 40 days. It would therefore seem that under optimum conditions the length of early oogenesis up to stage 2 is somewhat longer than later oogenesis (stages 2-6). It is not knownhow long stage 6 oocytes remain in an unovulated adult ovary, but resorption of larger oocytes, stages 3-6 (atresia), occurs in starved and unhealthy animals (Dumont, 1972) and Colman (1974) reported a value of 2% atretic oocytes in the healthy animals used in his study.

During oogenesis there is an intense accumulation of cellular components which are essential for future embryonic growth and

Table 1-1 Characteristics of the various stages of oocyte development in Xenopus laevis according to Dumont (1972)

Stage	Dia (μ)	RNA (ng)	Total Oocytes	General Appearance	Yolk	Nucleus & Nucleoli	Chromosomes
1	50 to 300	40 to 125	n.d.	Transparent cytoplasm; nucleus and mitochondrial mass clearly visible.	None	Nucleus centrally located, contour smooth. Nucleoli formation begins in late stage 1 & continues in stage 2	Zygotene, Pachytene and very early diplotene.
2	300 to 450	125 to 400	45%	Cytoplasm translucent early, white and opaque later; nucleus & mitochondrial mass visible in early stage 2.	Small Platelets & lipid in periphery		Diplotene and early lampbrush
3	450 to 600	400 to 1500	13%	Pigment forms - light brown early, uniformly blackish-brown late; lightly pigmented at area of attachment to ovary	Loosely packed platelets surrounded by lipid fill cytoplasm by end of stage 3	Nuclear membrane sacculated; Nucleoli increase in size	Maximum lampbrush
4	600 to 1000	1500 to 3000	15%	Animal and Vegetal hemispheres become differentiated	Progressive accumulation	Nucleus located near animal pole	Lampbrush retract
5	1000 to 1200	3000 to 4000	10%	Hemispheres clearly delineated at equator; animal hemisphere appears light brown	Decreasing accumulation	Nucleoli decrease in size & number & move to centre of nucleus.	Condensed in centre of nucleus
6	1200 to 1300	4000 to 4300	13%	Unpigmented equatorial band	Cytoplasm packed with platelets and lipid	Some nucleoli at vegetal pole of nucleus	

differentiation (Wischnitzer, 1966; Davidson, 1976). The stage 6 Xenopus oocyte has a volume about 200,000 times the volume of a Xenopus liver cell and much of the increase in size of the oocyte is due to the accumulation of yolk platelets (Wallace, 1972). Large numbers of ribosomes, mitochondria, RNP particles, soluble proteins and tRNA molecules also accumulate (Rosbash & Ford, 1974; Davidson, 1976; Webb & Smith, 1977), and as a result the fertilized egg can develop rapidly to a tadpole in about 5 days. Sufficient ribosomes are stockpiled during oogenesis to allow the anucleolate mutant of Xenopus laevis to develop into a swimming tadpole before death (Brown & Gurdon, 1964) and, in the absence of RNA synthesis, physically enucleated and actinomycin D treated embryos can complete cleavage normally before developmental arrest (Wallace & Elsdale, 1963; Brachet et al., 1964). Also a number of maternal effect mutations exist in which the development of the embryo is blocked at a specific stage, but in which the block can be removed by injecting cytoplasm from non mutant eggs or oocytes (Briggs & Justus, 1968). Clearly, the developing embryo is dependent on many substances synthesized and stored during oogenesis.

This dependence of the embryo on maternal components is reflected in the dramatic change in macromolecular synthesis which occurs after fertilization. The oocyte can be considered to be a cell specialized for the accumulation and storage of various macromolecules and is in a suspended meiosis where no chromosomal DNA synthesis occurs (Gurdon, 1967) but rapid RNA and protein synthesis is observed at all stages (LaMarca et al., 1973; Darnbrough & Ford, 1976; Anderson & Smith, 1977). In contrast after fertilization, little RNA synthesis is detected for some time (Gurdon, 1968), DNA synthesis

soon begins at a very rapid rate and protein synthesis is also activated (Hinegardner et al., 1964; Graham & Morgan, 1966; Ecker, 1972; Shih, 1975). The increase in protein synthesis exceeds new message synthesis and must be due to the utilization of stored mRNA and other protein synthetic machinery (Humphreys, 1969, 1971; Mano & Nagano, 1970).

Obviously, knowledge of the pattern and control of macromolecular synthesis during oogenesis will be crucial to the understanding of the differentiation and development of the embryo. Many of the cellular components which are stockpiled during oogenesis are required for the rapid protein synthesis which occurs after fertilization and which appears to be programmed by the stored mRNA. Clearly the control of transcription and the processes of storage of the mRNA synthesized during oogenesis are fundamental problems of developmental biology about which little is known. As this investigation was aimed at the study of RNA synthesis and in particular mRNA synthesis during oogenesis, it is appropriate to review the current state of knowledge. Several different classes of RNA are known to be synthesized during oogenesis and these are discussed in turn.

(b) Transfer RNA

There are about 8-10,000 genes coding for at least 43 basic tRNA molecules in the haploid genome of Xenopus laevis and there appear to be different numbers of genes for certain tRNA species (Clarkson et al., 1973). Transfer RNA is a major transcription product in young oocytes (stages 1-2) (Ford, 1971, 1972; Thomas, 1974), and synthesis of tRNA continues throughout oogenesis but accounts for a much smaller percentage (less than 5%) of the total

RNA synthesis in stage 6 oocytes (LaMarca et al., 1973; Colman, 1974) largely due to the increase in rRNA synthesis. However, it is not known whether the rate of tRNA synthesis changes during oogenesis.

Much of the tRNA synthesized during previtellogenesis is very stable (Ford et al., 1977) and initially it appears to be stored along with 5S RNA complexed with protein in a 42S RNP particle (Ford, 1972). Later in oogenesis, few 42S RNP particles are observed, the 5S RNA having been released and incorporated into ribosomes, and the tRNA being bound in the cytoplasm (Ford, 1971; Mairy & Denis, 1972).

In Xenopus, the chromatographic behaviour of oocyte tRNA differs from that of somatic cell tRNA and this may be due to modifications to the tRNA since the nucleotide sequence of at least one tRNA is the same in both cell types (Wegnez et al., 1975). Differences in chromatographic behaviour between tRNA from small oocytes and full grown oocytes have also been reported (Denis et al., 1975) and it is not known whether a similar explanation applies in this case.

(c) 5S RNA

5S RNA is transcribed throughout oogenesis from highly repetitive DNA, there being 20-25,000 copies of the 5S RNA genes present in the haploid genome of Xenopus laevis (Brown & Weber, 1968). The pattern of 5S RNA synthesis during oogenesis in Xenopus is similar to tRNA synthesis in that 5S RNA constitutes a major proportion of the total RNA synthesis in stage 1 oocytes (Ford, 1972; Thomas, 1974) but is hardly detectable in stage 6 oocytes (LaMarca et al., 1973; Colman, 1974; Brown & Gurdon, 1977). Again this RNA species is very stable (Ford et al., 1977) and is initially stored in the cytoplasm complexed with protein and tRNA in 42S RNP particles (Denis & Mairy, 1972; Ford, 1972). When 18S and 28S rRNA synthesis accelerates at about the

beginning of vitellogenesis the 5S RNA is released from these particles and is incorporated into the oocyte's growing number of ribosomes (Mairy & Denis, 1972). Here again it is not known whether there is a change in the rate of 5S RNA synthesis during oogenesis.

As for tRNA, the chromatographic behaviour of 5S RNA in Xenopus oocytes differs from Xenopus somatic cell 5S RNA (Denis et al., 1972, 1975; Ford & Southern, 1973), but in this case sequence differences are the cause. The oocytes appear to utilise all the 5S RNA genes while somatic cells express only a fraction which differ from the oocyte specific sequences in up to 7 of the 120 nucleotides in these molecules (Ford & Southern, 1973; Brownlee et al., 1974).

(d) 18S and 28S ribosomal RNA

In the oocytes of many organisms amplification of the gene sets for 18S and 28S rRNA occurs during early oogenesis (Davidson, 1968) and in amphibia this process occurs mainly at the pachytene stage. When functional the amplified rDNA is circular in form and is located in some 1500 extrachromosomal nucleoli (Miller, 1966; Hourcade et al., 1973). In the Xenopus oocyte the total number of ribosomal gene sets is 3-5 x 10^3, each set containing some 450 copies of the rRNA genes. Thus the total number of rRNA genes per oocyte in Xenopus is 1.5-2.5 x 10^6, which is about 30 pg of extra-chromosomal DNA (Perkowska et al., 1968). In the absence of rDNA amplification the oocyte, synthesizing ribosomes at a rate equal to that in Hela cells, would require about 1000 years to accumulate the number present in the stage 6 Xenopus oocyte (Ford, 1967).

Despite this amplified rDNA being present in the previtellogenic diplotene oocytes of amphibia, little rRNA synthesis occurs compared

with later stages (Ford, 1972; Thomas, 1974; Scheer et al., 1976). Thus coordinate synthesis of 5S RNA with 18S and 28S RNA does not occur in the previtellogenic oocyte. In the newt Triton alpestris, Scheer et al. (1976) have correlated this low rate of synthesis with low or non existant packing of RNA transcripts on nucleolar DNA. In midvitellogenic oocytes of Triton and Xenopus 90-95% activity is observed on the nucleolar genes (Miller & Beatty, 1969b). It therefore appears that activation of rRNA synthesis must occur at some time after amplification and this may be due to gradual unfolding of the rDNA from its tightly coiled post amplification form (Ford, 1972).

The size of the initial rRNA transcript in Xenopus is $2.5\text{-}2.6 \times 10^6$ daltons (40S) and both 18S and 28S rRNA are cleaved from this precursor (Loening et al., 1969). A major intermediate in the production of 28S RNA is the nucleolar 30S RNA species and the process of maturation for rRNA seems similar in Xenopus laevis and Pleurodeles poireti (Loening et al., 1969; Wellauer and Dawid, 1974; Denoulet et al., 1977).

Ribosomal RNA accounts for most (over 90%) of the stable RNA synthesized in oocyte stages 3, 4, 5 and 6 (Davidson et al., 1964; LaMarca et al., 1973; Colman, 1974) and some estimates of the actual rate of stable RNA synthesis have been made. Scheer (1973) showed that 3.8 µg of cytoplasmic rRNA accumulated in vivo in 38 days in rapidly growing Xenopus laevis oocytes in females stimulated with HCG. This is an average rate of accumulation of rRNA of 4.2 ng/oocyte/hour. LaMarca et al. (1973) were able to estimate the rate of synthesis of stable RNA in stage 3 and stage 6 oocytes of Xenopus laevis by measuring the incorporation of radioactive guanosine into RNA in vitro while changes in the GTP precursor specific activity were monitored.

These investigators showed that similar rates of RNA synthesis of about 1 ng/oocyte/hour occured in both maximal lampbrush stage oocytes (stage 3) and in full grown oocytes (stage 6) and they suggested that previous studies which implied low or non existant *in vivo* rates of RNA synthesis in stage 6 *Xenopus laevis* oocytes (Brown & Littna, 1964a, b; Brown, 1966) were probably due to very low precursor specific activities which were not determined. Both *Rana pipiens* and *Triton alpestris* full grown oocytes have been shown to readily incorporate labelled precursor into RNA (Smith & Ecker, 1970; Scheer et al., 1976).

A further study measuring *in vitro* rates of RNA synthesis in *Xenopus laevis* again based on precursor pool specific activity measurements showed that HCG stimulation had no substantial effect on the incorporation of injected GTP into RNA in stage 6 oocytes. The average rate of synthesis of stable RNA in several experiments was about 2 ng/oocyte/hour. Using similar conditions Anderson & Smith (1977) obtained an average *in vitro* rate of stable RNA synthesis in micro-injected stage 6 *Xenopus laevis* oocytes of 0.73 ng/oocyte/hour. At these *in vitro* rates the accumulation of 4 μg of rRNA would take 2-6 months.

In *Triton alpestris* it has been shown that the rate of rRNA synthesis in full grown oocytes is only about 13% of that measured during midvitellogenesis and this observation is correlated with an 85% reduction in the number of transcripts observed on the nucleolar genes between mid and late vitellogenesis (Scheer et al., 1976). On the other hand Davis & Wilt (1972) have reported that during the final one third of oogenesis in the worm *Urechis caupo* the rate of RNA synthesis is 4-5 fold greater than at earlier times.

Although as in *Triton* the rate of stable RNA synthesis in stage 3 *Xenopus* oocytes increases dramatically from the very low rate of

synthesis in smaller oocytes, the results obtained *in vitro* by LaMarca et al. (1973) suggest that in *Xenopus* the rate of stable RNA synthesis remains roughly constant from stage 3 to stage 6. However, Rogers & Browder (1977a) have shown that in small *Rana pipiens* oocytes, culture *in vitro* causes an apparent acceleration in lampbrush chromosome morphology correlated with RNA synthesis and thus observations made *in vitro* may not apply *in vivo*.

As pointed out by Colman (1974), if the rates of stable RNA synthesis measured *in vitro* apply *in vivo* in stage 6 oocytes, then in animals which have not ovulated for several months, there should be an accumulation of RNA in excess of the 4-5 μg present in stage 6 oocytes, unless some other factor is operating. In this connection Anderson & Smith (1977) estimate the minimum half-life for rRNA in stage 6 *Xenopus* oocytes to be 144 days using their measured rates of synthesis and assuming that 3.2 μg of rRNA present represents a steady state amount. Although this half life greatly exceeds that measured *in vivo* by Leonard & LaMarca (1975) of 9-30 days it still suggests that in unovulated animals either the whole pool of ribosomes accumulated during oogenesis in *Xenopus* turns over at least once before the completion of oogenesis, or a fraction of the ribosomes (perhaps the newer ones or those engaged in protein synthesis) turn over more rapidly. Clearly the significance of rRNA synthesis in stage 6 *Xenopus laevis* oocytes is not understood.

(e) Mitochondrial RNA

Webb & Smith (1977) measured the mitochondrial DNA content of Xenopus laevis oocytes at various stages of oogenesis by hybridization with radioactive cRNA. They found that accumulation of mtDNA proceeds until stage 4 when a steady state level of about 4.28 ng/oocyte is attained. This quantity of mtDNA is about 300 times the chromosomal DNA content (12.5 pg) and is sufficient for about 1.8×10^8 mitochondrial genomes (Davidson, 1976). This value is similar to the 3.8 ng obtained in an earlier study using Xenopus laevis eggs (Chase & Dawid, 1972) in which the rate of mitochondrial rRNA synthesis in early embryos was estimated to be about 30 pg/hour/embryo. Webb et al. (1975) estimated the minimum rate of total mtRNA synthesis in stage 6 Xenopus laevis oocytes to be 21-45 pg/oocyte/hour, mitochondrial rRNA accounting for about 30% of this and these authors found no evidence for a change in the rate of mtRNA synthesis during progesterone induced maturation in either Xenopus laevis or Rana pipiens. Therefore, since the number of mitochondrial genomes present is constant from about stage 4 the rate of mtRNA synthesis is probably little altered from stage 4 oocytes until about gastrulation when a burst of mitochondrial rRNA synthesis has been shown to occur (Chase & Dawid, 1972).

Apart from the 30% mitochondrial rRNA, Webb et al. (1975) showed that in oocytes from Xenopus laevis 4S RNA accounted for about 10% of the measured mtRNA synthesis and heterogeneous RNA accounted for about 50%. At the tailbud stage of Xenopus laevis embryogenesis about 15 species of tRNA and some heterogeneous RNA species are synthesised by the mitochrondia (Chase & Dawid, 1972).

In sea urchins mtRNA is synthesised in enucleated egg cytoplasm (Craig, 1970) which can be severely inhibited by ethidium bromide (Craig & Piatigorsky, 1971). Here again mitochondrial rRNA, mitochondrial tRNA and heterogeneous RNA were observed (Chamberlain & Metz, 1972).

Devlin (1976) identified 8 distinct species of poly(A)$^+$RNA synthesized by mitochondria in enucleated egg fragments in the sea urchin, together with mitochondrial tRNA and rRNA and the pattern of synthesis remained the same throughout development. Since all these species would account for 96% of the mitochondrial genome Devlin concluded that the whole of the mitochondrial genome is expressed throughout embryogenesis. Poly(A)$^+$RNA has also been found in mamalian and insect mitochondria (Perlman et al., 1973) which has been shown to contain about 8 distinct species and has a poly(A) length of about 50-60 A residues (Hirsch et al., 1974).

It is therefore clear that in any study of mRNA metabolism during oogenesis in Xenopus, consideration must be given to the possibility that a significant proportion of the mRNA present and being synthesized may be mitochondrial. In this connection Webb et al. (1975) presented preliminary evidence that as much as 30% of the radioactive mitochondrial RNA from stage 6 Xenopus laevis oocytes contained poly(A).

(f) Heterogeneous nuclear RNA

There is a considerable body of evidence which shows that hnRNA similar to that of somatic cells is synthesized throughout oogenesis. Heterogeneous RNA species, some of very large size, have been observed in sea urchin oocytes (Sconzo et al., 1972), mouse oocytes (Bachvarova, 1974; Jahn et al., 1976), the urodele Triturus (Sommerville, 1973; Malcolm & Sommerville, 1974) and in various oocyte stages of Xenopus laevis (Davidson et al., 1964; Mairy and Denis, 1971; Thomas, 1974; Anderson & Smith, 1977). Thomas (1974) found that after 24 hours of labelling about 25% of the total radioactivity present in RNA in stage 1 Xenopus laevis oocytes was in heterogeneous RNA in the nucleus. Much of this RNA sedimented faster than 40S but in this study insufficient precautions were taken to prevent aggregation of the RNA molecules.

A unique feature of germ-line cells are the lampbrush chromosomes and investigations during the period when the oocyte chromosomes have assumed this form have shown that the synthesis of RNA with the properties of hnRNA occurs on these chromosomes (Sommerville, 1973; Angelier & Lacroix, 1975). Roughly 5-10% of the chromosomal DNA is extended into many pairs of loops (Gall, 1955; Vlad & MacGregor, 1975), in Triturus about 20,000 loops per 4C chromosome set, and radioautograph experiments have shown that newly synthesized RNA and protein can be observed along the whole length of most loops (Gall & Callan, 1962). The number of loops per unit length of chromosome is roughly constant and thus species with large C values accommodate the extra DNA in larger lampbrush chromosomes with larger loops (Gall, 1955; Callan, 1963; Vlad & MacGregor, 1975). By using fluorescein-linked antibodies to nuclear sap RNP particles Scott &

Sommerville (1974) observed only about 10 loops reacting and consequently the RNP, and presumably the DNA, content of the loops must be specific. The sequences of DNA present in the loops are interspersed repetitive and single copy sequences (Davidson et al., 1973; MacGregor et al., 1976; Sommerville & Malcolm, 1976). By visualization in the electron microscope the loops contain one or a few units of transcription separated by spacer regions where no transcription occurs and the polarity (direction of transcription) may be reversed within a loop (Angelier & Lacroix, 1975). From these studies it is clear that the average length of the RNA transcripts in urodeles (up to 10^5 nucleotides) is very much larger than in anurans such as Xenopus.

For Triturus, it has been shown that the large RNA transcripts made on the lampbrush chromosomes are released into the nuclear sap as RNP particles composed of about 97% protein and 3% RNA (Sommerville, 1973; Malcolm & Sommerville, 1974). These nuclear RNP particles are similar to those observed in typical somatic cells which contain hnRNA (Pederson, 1974; Kumar and Pederson, 1975). The RNA isolated from these particles appears to have sedimentation values in the range 40-100S, a low GC base composition and a high uridylic acid content (Sommerville, 1973; Malcolm & Sommerville, 1974; Sommerville & Malcolm, 1976).

These observations on the lampbrush chromosomes of oocytes which show that a class of RNA is synthesized with similar properties to the hnRNA of somatic cells, part of which is believed to be the nuclear precursor of mRNA (Lewin, 1974), have led to the conventional view that lampbrush chromosomes are specialized structures for the synthesis of the maternal mRNA which is accumulated during oogenesis.

The actual rate of nuclear RNA synthesis based on specific activity measurements in stage 6 oocytes of Xenopus laevis has recently been reported (Anderson & Smith, 1977). The nuclear RNA synthesized was separated on gels into 3 classes: >40S heterogeneous RNA, 4-40S heterogeneous RNA and 40S rRNA precursor. The half-life of the >40S RNA was about 30 minutes and it was synthesized at an initial rate of 0.75 ng/oocyte/hour which was about half the initial rate of synthesis of the 4-40S RNA which had a half-life of about 5 hours. The base composition of the >40S RNA was low in GC content and had an elevated uridylic acid content. This rapid rate of hnRNA synthesis in stage 6 oocytes which is nearly three orders of magnitude greater than in somatic cells was reported to be comparable to rates of hnRNA synthesis measured in stage 3 Xenopus laevis oocytes and therefore Anderson & Smith (1977) suggested that the high RNA polymerase packing observed in the lampbrush chromosomes stages holds throughout later oogenesis and explains the high rate of synthesis relative to somatic cell nuclei.

Based on these observations and also those made on stage 1 Xenopus laevis oocytes (Thomas, 1974) it must be concluded that because hnRNA is synthesised both before and after the lampbrush chromosome stage, these structures are not necessary for the production of hnRNA and the similar rate measurements between stage 3 and stage 6 oocytes suggests that these chromosomal forms are also not required for the extremely rapid synthesis of hnRNA which occurs.

In Triturus Sommerville & Malcolm (1976) have shown that the hnRNA synthesized on the lampbrush chromosomes contains both repetitive sequence transcripts and it has been shown in earlier work (Davidson et al., 1966; Crippa et al., 1967) that repetitive sequence transcripts.

accumulate during oogenesis in Xenopus and are passed on to the embryo. These sequences cannot be completely accounted for by histone mRNA and tRNA and may represent either mRNA transcribed from repetitive structural genes or repetitive sequences in hnRNA (Davidson, 1976).

The existence of poly(A) in hnRNA in somatic cells is well documented (Lewin, 1974; Edmonds et al., 1976; Kinniburgh et al., 1976). Poly(A) constitutes roughly 0.5-1.0% of the total RNA and is present on about 20% of the hnRNA molecules (Lewin, 1974). Since those hnRNA molecules which contain poly(A) are believed to be precursors to the cytoplasmic mRNA in somatic cells poly(A) sequences have been looked for in oocyte hnRNA. As poly(A) is quite resistant to digestion by RNAses A + T_1, but susceptible to digestion by RNAse T_2, Rogers & Browder (1977b), using Rana pipiens have sequentially subjected ^{3}H-adenosine labelled lampbrush stage oocyte sections to these enzymes. In conditions where nucleolar RNA and ^{3}H-uridine labelled RNA show no RNAse T_1 resistance their autoradiographic measurements show that about 6% of the ^{3}H-adenosine incorporated into nucleoplasmic RNA is resistant to RNAse T_1 but sensitive to RNAse T_2 and is presumably poly(A). If the base composition of the hnRNA is 25% adenylic acid then the percentage poly(A) in the nucleoplasmic RNA will be about 1.5%.

In a different study by Denoulet et al. (1977) using the urodele Pleurodeles poireti radioactive RNA was prepared from isolated nuclei of lampbrush stage oocytes and subjected to gel analysis before and after poly(U) sepharose chromatography. Although no direct demonstration of poly(A) was given about 8-15% of the >40S hnRNA was retained on poly(U) sepharose in greater than 90% formamide and little hnRNA <40S was present. Even allowing 200 A residues per

molecule and a minimum size of 40S the percentage poly(A) in hnRNA of Pleurodeles is only 0.2-0.4%.

This difference between these species is probably related to the C value since the hnRNA transcripts in Pleurodeles, of up to 10^5 nucleotides (Angelier & Lacroix, 1975) are likely to be much longer than those of Rana. Thus approximately the same number of molecules may have poly(A) sequences in both anurans and urodeles, however, direct demonstration that polyadenylated hnRNA is a precursor of mRNA in oocytes has not been made.

(g) Messenger RNA

Although oocytes contain mitochondrial mRNA (Section (e)) and mRNA not stored for use in embryogenesis, the most important class of mRNA present in oocytes, both quantitatively and in terms of development, is maternal mRNA (Davidson, 1976). Maternal mRNA appears to be a general feature of oocytes, being accumulated during oogenesis and not being utilized for protein synthesis until during maturation and after fertilization when this stored mRNA is mobilized and used to direct protein synthesis in the developing embryo (Smith & Ecker, 1965, 1969; Ecker, 1972).

Historically, the demonstration of maternal mRNA was achieved by studying protein synthesis in enucleated eggs undergoing maturation, fertilization or early development. Protein synthesis occured which was quantitatively and qualitatively similar to that of control nucleated samples (Tyler, 1965; Clement & Tyler, 1967; Ecker et al., 1968; Ecker, 1972) and was not due to mitochondrial protein synthesis (Craig & Piatigorsky, 1971). Similar experiments in which embryos were treated with actinomycin in order to block new mRNA synthesis

showed that protein synthesis could continue for some time and the existence of maternal mRNA was inferred (Gross & Cousineau, 1964; Gross, 1967; Raff et al., 1971).

Finally, more direct proof of maternal mRNA has been shown by several groups of workers who extracted RNA from various oocytes or eggs, some 2-4% of which was template active in a cell free translation system such as the E. coli, rat liver or wheat germ systems (Maggio et al., 1964; Slater & Spiegelman, 1966; Davidson et al., 1966; Cape & Decroly, 1969). Stored maternal mRNA thus seems to be a common constituent of animal oocytes. Further evidence of the function of this mRNA has been reported by many workers (Infante & Nemer, 1967; Mano, 1971; Humphreys, 1971) who have shown that both the number of ribosomes and the amount of mRNA in polysomes increases significantly after fertilization and consequently much of the stored maternal mRNA must have been mobilized.

More recent investigations have relied on the observations that most mRNA molecules contain a 3' poly(A) sequence (Kates, 1970; Edmonds et al., 1971; Jelinek et al., 1973; Greenberg, 1975). In both sea urchin and Xenopus oocytes the poly(A) tracts are about 100 A residues long and are attached to heterogeneous RNA molecules with a number average size of about 2000 nucleotides and account for 0.4-0.8% of the total RNA (Wu & Wilt, 1973, 1974; Rosbash & Ford, 1974). Although these values of the percentage mRNA are lower than those obtained from translation experiments much data suggests that about half of the mRNA in unfertilized sea urchin eggs is poly(A)$^-$RNA (Ruderman & Pardue, 1977) and poly(A)$^-$mRNA is known to be synthesized later in sea urchin development (Nemer et al., 1974, 1975; Fromson & Duchastel, 1975;

Fromson & Verma, 1976). Further, the content of poly(A) increases up to 2 fold within several hours of fertilization in sea urchins (Slater et al., 1972, 1973; Wilt, 1973) and appears to be due to cytoplasmic polyadenylation of pre-existing maternal mRNA. Thus, the total mRNA content of unfertilized eggs must be about 2%, consisting of a mixture of poly(A)$^+$ and poly(A)$^-$RNA.

By hybridization with ^{3}H-poly(U), Rosbash & Ford (1974) showed that poly(A)$^+$RNA in Xenopus oocytes had the characteristics of mRNA given above and also that the amount of poly(A) present per oocyte remained roughly constant from stage 2 oocytes to stage 6 oocytes. This poly(A)$^+$RNA is template active in the wheat germ system yielding about 40 prevalent proteins, yet no significant differences in translation products were apparent using poly(A)$^+$RNA from oocyte stages 1 to 6 or using oocyte poly(A)$^-$RNA despite the fact that changes in protein synthesis occur in vivo (Darnbrough & Ford, 1976).

Over 90% of the poly(A)$^+$RNA in Xenopus oocytes is present in RNP particles which sediment heterogeneously, but more slowly than ribosomes (Rosbash & Ford, 1974). In sea urchin oocytes the RNA in cytoplasmic RNP particles is template active in cell free systems, (Gross et al., 1973; Slater et al., 1973) and in competition hybridization experiments this RNA contains more than 95% of the total oocyte histone mRNA (Farquhar & McCarthy, 1973; Lifton & Kedes, 1976). Some 0.2-0.4 ng of histone mRNA may be accumulated during oogenesis in Xenopus (Adamson & Woodland, 1977) and about half of this is polyadenylated (Woodland, personal communication) thus histone mRNA would account for about 0.5% of the 40 ng of total poly(A)$^+$RNA in Xenopus oocytes and as yet this is the only specific mRNA identified in this species.

Very recently Ford et al. (1977) have demonstrated, by injecting radioactive uridine and guanosine into immature Xenopus laevis, that the radioactivity incorporated in vivo into poly(A)$^+$RNA in these stage 1 oocytes is retained for 18 months during which time the ovary grows and produces normal stage 6 oocytes. This data and that above strongly suggests that in Xenopus some maternal poly(A)$^+$mRNA is synthesized during stage 1 and stored, and since little or no accumulation of poly(A)$^+$RNA occurs from stage 2 to stage 6 oocytes, any further synthesis of poly(A)$^+$RNA must be balanced by turnover in oocyte stages 2 to 6.

Anderson & Smith (1977) have recently reported that about 15% of the total stable RNA (half-life $>$ 90 hours) synthesized in vitro by stage 6 Xenopus laevis oocytes is heterogeneous in size (4-40S) and accumulates in the cytoplasm at a rate of about 0.1 ng/oocyte/hour. These authors suggest that some of this RNA represents maternal mRNA which is added to the stockpile of transcripts. If much of this class of RNA is polyadenylated then it would be hard to reconcile this observation with that of Rosbash & Ford (1974) above.

The information reviewed here is paradoxical in that it suggests that the accumulation of poly(A)$^+$mRNA has occurred by stage 2 and that the accumulated mRNA appears to be very stable. On the other hand stage 6 oocytes appear to synthesize apparently stable heterogeneous RNA and in Davidson's review (1976) unpublished data of Doleki & Smith is presented which shows that poly(A)$^+$RNA is synthesized in both stage 3 and stage 6 oocytes of Xenopus. However, it is not known what proportion if any of the poly(A)$^+$RNA synthesized in later stages of oogenesis is stable but only if most of it is not stable are the above reports easily reconciled.

One of the main objectives of this thesis was to try to resolve this paradox by determining whether or not poly(A)$^+$RNA was synthesised in large oocytes. If poly(A)$^+$RNA was observed it would be important to establish its stability and to estimate what proportion of the maternal poly(A)$^+$mRNA it might represent.

(h) Experimental system

The experiments in Chapters 3, 4 and 5 describe results obtained by *in vitro* incubation of *Xenopus* ovary and oocytes in radioactive media. Usually *in vitro* incubation of solid tissue is associated with many difficulties since the supply of blood with its essential metabolites has been cut off. Attempts to overcome the low penetration of metabolites (in particular oxygen) often involved slicing the tissue, which in itself causes further difficulties due to cell damage and hydrolytic enzyme release. Since it is not obvious what effects removal and incubation *in vitro* may have on the molecular processes occuring within a tissue, observations made using these conditions should be interpreted with caution.

Although the same arguments no doubt apply to ovary and oocytes incubated *in vitro* this tissue probably has advantages over others. Being a diffuse tissue consisting of large single cells (oocytes) surrounded by a thin layer of follicle cells the problems of metabolite penetration must be considerably less critical than with solid tissue and cell damage on removal is minimal. Since the oocyte itself contains large pools of metabolites, and cell organelles for use during development (Davidson, 1976) it is not surprising that oocytes incubated in a simple salt medium appear to remain healthy, as judged biochemically, for 1-4 weeks (Colman, 1974; Gurdon, 1974).

Since ovary and oocytes survive well after interruption of the blood supply, it is generally assumed that observations made *in vitro* are applicable *in vivo*, but this may not be the case and indeed some morphological changes in the oocytes of *Rana pipiens* have been detected during culture *in vitro* (Rogers & Browder, 1977a).

In Chapter 4 experiments involving microinjection of stage 6 oocytes of *Xenopus laevis* are described. In the first instance this operation may seem somewhat traumatic for the oocyte. A sharp piece of glass is forced through the layer of follicle cells, often subjecting the oocyte to a considerable degree of squashing, the cell membrane is damaged and a volume of solution, usually buffered with unphysiological compounds is released into the cytoplasm, some leakage of cell contents often occuring. Despite these manipulations, several groups of workers have shown that microinjected oocytes survive and synthesize protein and RNA for up to 14 days (LaMarca et al., 1973, 1975; Colman, 1974; Gurdon, 1974). Indeed Colman (1974) demonstrated that protein and RNA synthesis is unaffected by pricking the oocyte or injection of 30 nl of various solutions and this agrees with the observations of Gurdon (1974) who has performed nuclear transfer experiments, which demonstrate that healthy tadpoles and frogs can develop from microinjected eggs. It is therefore considered that microinjection causes no significant and reproducable damage to the oocyte and that results gained using this technique are valid.

(i) Experimental approach

The primary objective of this investigation was to gain information on the synthesis of mRNA during oogenesis in Xenopus laevis. As has been pointed out 2 major classes of mRNA are known poly(A)$^+$ and poly(A)$^-$mRNA. While both classes can usually be obtained from somatic cell polysomes (Lewin, 1974; Greenberg, 1975), in Xenopus oocyte polysomes are only easy to prepare from early stages and much of the mRNA in larger oocytes is not associated with polysomes (Rosbash & Ford, 1974). Consequently, in this study poly(A)$^+$RNA has been isolated by its affinity for oligo(dT)-cellulose using conditions similar to those of Cabada et al. (1977). Therefore the poly(A)$^+$RNA (oligo(dT) bound RNA) under investigation must contain tracts of A residues longer than 30 bases (Cabada et al., 1977). However it should be realized that the oligo(dT) void RNA may contain heterogeneous RNA with short poly(A) tracts (sometimes called poly(A)$^-$RNA in the literature) and/or heterogeneous RNA with no poly(A) (true poly(A)$^-$RNA) about which little firm data has been gained.

In Chapter 3 preliminary incubation experiments are described which established that heterogeneous poly(A)$^+$RNA was synthesized in stage 6 Xenopus oocytes incubated in vitro, but problems in the isolation of poly(A)$^+$RNA and in the preparation of nuclei had to be overcome before the cellular distribution and kinetics of synthesis could be determined.

The technique of oocyte microinjection used in Chapter 4 on stage 6 oocytes facilitated the study of the kinetics of synthesis and quantitation of the RNA made, and kinetic curves for the synthesis of total RNA and poly(A)$^+$RNA in the nucleus and cytoplasm were also obtained. Quite different kinetic curves were obtained when labelling

nuclear poly(A)$^+$RNA with ATP and GTP and this was due to poly(A) synthesis. Similar kinetic curves for the synthesis of poly(A)$^+$RNA labelled with GTP were obtained with enucleated and intact oocytes but ethidium bromide treatment of intact oocytes selectively inhibited poly(A)$^+$RNA synthesis. These observations strongly suggested that a large proportion of the poly(A)$^+$RNA synthesized in stage 6 oocytes was mitochondrial and further attempts were made to verify this.

Chapter 5 presents a similar study on stage 1 oocytes. The kinetic curves of incorporation of radioactive nucleoside into both total RNA and poly(A)$^+$RNA by previtellogenic ovary were similar to those for stage 6 oocytes and using an enzymic separation to remove the follicle cells most of the incorporation was shown to be due to the oocytes. On sucrose gradients the proportions of the various RNA species synthesized by stage 1 oocytes were very different from those of stage 6 oocytes and some properties of the poly(A)$^+$RNA synthesized showed differences between these oocyte stages.

In Chapter 6 the development of a method for measuring actual specific activities of NTP precursors based on an RNA polymerase assay is reported and rates of RNA synthesis were measured for both stage 1 and stage 6 oocytes by using this technique. As a result it was found necessary to apply a correction to the estimated rates of RNA synthesis of Chapter 4.

In the final Chapter the results of this investigation are discussed in terms of the present state of knowledge of RNA synthesis during oogenesis.

CHAPTER 2

Materials and Methods

MATERIALS

General reagents were from BDH and were analar grade wherever possible. TCA, sucrose and 2-mercaptoethanol were from Koch-Light; DTT, spermine, Hepes, Tris, SDS and NTPs were all from Sigma; DEP and TNS were from Eastman Kodak Company; dextran sulphate from Pharmacia and MS222 from Sandoz.

Antibiotics:

Gentamycin sulphate was from Sigma; streptomycin sulphate from Glaxo and sodium benzo penicillin was a gift from Joan Fleming.

Enzymes:

Bovine pancreatic RNAse Type 1-A; RNAse T_1 from Aspergillus orzyae Grade III; bovine pancreatic DNAse Type 1; collagenase from Cl. histolyticum Type 1 and hyaluronidase from ovine testes Type II were all from Sigma; RNA polymerase core enzyme from E. coli was a gift from Kevin O'Hare.

Radiochemicals:

Radiochemicals were all obtained from the Radiochemical Centre, Amersham.

(8-^{3}H) - guanosine aqueous solution 18 Ci/mmol Batch 58

(2-^{3}H) - adenosine aqueous solution 21 Ci/mmol Batch 11

(5-^{3}H) - uridine aqueous solution 28.1 Ci/mmol Batch 108

(8-^{3}H) - GTP ammonium salt 11.0 Ci/mmol Batch 14

(2-^{3}H) - ATP ammonium salt 16.4 Ci/mmol Batch 18

(5-^{3}H) - UTP ammonium salt 13.6 Ci/mmol Batch 39

α-^{32}P - GTP ammonium salt 9.2 Ci/mmol Batch 768

α-^{32}P - ATP triethylammonium salt 156 Ci/mmol Batch 768

α-^{32}P - ATP triethylammonium salt 107 Ci/mmol Batch 761

Synthetic polynucleotides:

Oligo (dT)-cellulose grade T-3 was from Collaborate Research Inc.; poly [d(A-T)] alternating co-polymer was from Sigma.

Animals:

Mature Xenopus laevis toads were obtained from the South African Snake Farm, Fish Hoek, S. Africa. Immature Xenopus laevis toads were either from the same source or were laboratory reared animals.

METHODS

(a) Buffers

The following buffer solutions were commonly used, all stored at 2°C, and sterilized where possible with DEP or by autoclaving.

MBX (modified Barth X)	NaCl	88 mM
	KCl	1 mM
	$Ca(NO_3)_2$	0.33 mM
	$CaCl_2$	0.41 mM
	$MgSO_4$	0.82 mM
	$NaHCO_4$	2.4 mM
	HEPES, pH 7.5	10 mM
gentamycin, streptomycin & penicillin each		0.01 g/l

SM (shrinking medium)	KCl	0.5 M
	NaCl	0.1 M

GVIM (germinal vesicle isolation medium)	KCl	100 mM
	NaCl	20 mM
	$MgCl_2$	2 mM
	$CaCl_2$	0.8 mM
	PVP	2%
	HEPES, pH 7.0	10 mM
	dextran sulphate	50 µg/ml
	spermine	50 µg/ml
	2-mercaptoethanol	1 mM

NETS (RNA buffer)	NaCl	100 mM
	EDTA	1 mM
	Tris HCl, pH 7.5	10 mM
	SDS	0.5%
Modified Kirby Buffer (RNA extraction buffer)	EDTA	10 mM
	SDS	0.5%
	TNS	1.0%
	PAS	6.0%
	NaCl	1.0%
	phenol/cresol	6.0%
	Tris HCl, pH 9.0	0.1 M
Phenol/Cresol	phenol (redistilled)	1000 g
	m-cresol	140 ml
	8-hydroxyquinoline	1 g
	water	100 ml

Oligo (dT)-Cellulose Buffers:

Binding buffer	NaCl	0.4 M
	EDTA	1 mM
	SDS	0.1%
	Tris HCl, pH 7.5	10 mM

Elution buffer is binding buffer without NaCl

SSC (poly(U) hybridization buffer)	NaCl	150 mM
	Sodium citrate	15 mM

(b) Preparation of ovary and oocytes

With one exception all the mature Xenopus laevis toads used in this study had been laboratory maintained on a 12 hour light/dark cycle in tanks at 20°C and fed twice weekly for a minimum of 2 months before killing. No animals which had been stimulated with HCG in their laboratory history were used.

Immature Xenopus laevis toads were either laboratory grown or obtained from South Africa and had been maintained under similar conditions with the exception that they were fed three times weekly. No differences were observed between laboratory reared and wild animals except that the former animals seemed to develop more slowly if grown in overcrowded conditions.

Ovaries were removed from pithed mature toads or from immature toads anaesthetized with MS222 and washed in several changes of MBX at 20°C to remove any blood. Mature ovary was stored in MBX at 20°C as thin sheets in petri dishes. Stored in this manner mature ovary would remain healthy for up to 7 days. Stage 6 oocytes were prepared from pieces of ovary by either manually plucking the oocytes with watchmakers forceps or when larger numbers were required, by dissociating the ovary in MBX containing 2 mg/ml collagenase and 20 mM EDTA for up to 30 minutes at 20°C. At the end of this treatment isolated oocytes were washed once with MBX containing 10% fetal calf serum and then several times with MBX. Healthy stage 6 oocytes were then selected. This enzymic procedure did not result in the removal of all the follicle cell layers which surround the oocyte (Colman, 1974).

(c) Incubation of ovary and oocytes

Batches of 20 stage 6 oocytes were incubated for various times in 50 or 100 μl MBX containing 8-^{3}H-guanosine or 2-^{3}H-adenosine at 100 μCi/ml then washed three times with unlabelled MBX and either immediately homogenized in modified Kirby buffer or treated as detailed below (sections (e) and (f)).

Up to 6 previtellogenic ovaries were cut into small pieces and incubated in 1.0 ml of MBX containing 5-^{3}H-uridine or 2-^{3}H-adenosine at 300 or 350 μCi/ml. After various times random pieces of ovary were selected and washed three times with unlabelled MBX and homogenized immediately in modified Kirby buffer or enzymically treated to remove follicle cells as detailed in section (e).

(d) Microinjection of stage 6 oocytes

The method of microinjection of oocytes used here is essentially that described by Gurdon (1974). Microinjection needles were prepared from hard glass capillary tubing by first hand pulling the heated glass to produce a uniform 4-5 cm section with an external diameter of about 300 μm and then by using a heated coil around the middle of this section to draw out, under gravity, a fine section with an external diameter of about 20 μm. The needle was nicked with a diamond and broken so that about 0.5 cm of the fine section remained attached to the 2-3 cms of intermediate section produced by the first hand pull. The tips of the microinjection needles were not micro-forged. Batches of needles prepared in this manner were siliconized and then washed by passing distilled water through the needles using a Perpex pump. Siliconization was found to improve the flow of liquid in the needles and eliminate wetting the glass.

The needle, filled from the wide end with liquid paraffin up to the middle of the intermediate section, was connected to a microsyringe by flexible polythene tubing which was also filled with liquid paraffin. The needle was held in a micromanipulator (Oxford Instruments) and filled by sucking in sample through the fine tip leaving an air space, to prevent mixing, between the paraffin meniscus and the sample meniscus. The needle was calibrated by expelling some of the sample so that it formed a drop hanging from the needle tip, and measuring its size on a stereomicroscope eyepiece scale. The drop volume was easily calculated from its measured diameter. Conveniently it was found that 5 divisions on the microsyringe vernier scale corresponded to 50 nl of sample and consequently needles were usually calibrated with divisions which corresponded to 50 nl painted onto the intermediate section of the needle with a fine paintbrush.

50 μCi of the radioactive NTP to be injected was dried down in a siliconized glass tube, redissolved in 1.1 μl of MBX and then taken up into the needle. Evaporation during this procedure reduced the volume to approximately 1 μl as measured in the calibrated needle and this was sufficient to microinject 50 oocytes with 20 nl (1 μCi) each.

Batches of 12, 20, or 25 oocytes were placed in an injection dish in MBX and injected under liquid. The injection dish was made by gluing two rectangular pieces of glass 1.5 mm thick onto a third piece with a gap of about 1.5 mm. The gap was closed at each end with smaller pieces of glass so forming a trough in which the oocytes could be placed and completely immersed in MBX. It was considered necessary to inject under liquid, despite the increased leakage which may have resulted because oocytes which had been subjected to drying during the

pooled oocytes were collected in a measured volume and a sample examined microscopically to determine the number of oocytes present and whether they were free of follicle cells. Preparations still contaminated with follicle cells were discarded.

(f) Preparation of germinal vesicles from stage 6 oocytes

Germinal vesicles were removed manually from stage 6 oocytes essentially by the method of Gall (1966). Oocytes from which the follicle layers had been removed, were placed in GVIM, and a small hole made in the animal poles using a fine platinum wire. Using watchmakers forceps, pressure was applied equatorially to the oocytes and their germinal vesicles gently squeezed out. Little, if any, cytoplasm remained attached to the germinal vesicles and if this could not be removed with forceps the germinal vesicle was discarded. Isolated germinal vesicles were immediately collected in a siliconized nuclear pipette (a pasteur pipette with the tip drawn out to a diameter of about 600 μm), placed in modified Kirby buffer and homogenized. The remaining cytoplasms were also immediately transferred to modified Kirby buffer and homogenized. It was also important to discard cytoplasm if intact germinal vesicles were not removed from them.

The method of enucleation used was identical, and care was needed to keep damage to the cytoplasmic membrane to a minimum if microinjection was to be successfully carried out.

(g) Extraction of RNA

Ovary fragments, oocytes, cytoplasms or germinal vesicles were homogenized in at least 5 volumes of modified Kirby buffer, with the addition of carrier RNA or labelled recovery control RNA if necessary, and the homogenate was then shaken with an equal volume of phenol/cresol mixture to form an emulsion. The emulsion was broken by centrifugation (10,000 rpm for 10 minutes in a Sorval HB4 rotor) and the aqueous phase was removed and similarly re-extracted with an equal volume of phenol/cresol mixture after readjusting the salt concentration by the addition of 1/10 volume of a solution containing 3 M NaCl, 10 mM EDTA, 100 mM Tris HCl, pH 9.0. If, after the first centrifugation, a clear interface was not obtained, then the organic phase was successively re-extracted with modified Kirby buffer until a clear interface was obtained, and all the aqueous phases were pooled prior to the second extraction. 2.5 volumes of ethanol at -20°C, were added to the second aqueous phase and the RNA allowed to precipitate overnight. The precipitate was collected by centrifugation at 10,000 rpm at 4°C for 15 minutes in a Sorval HB4 rotor, and traces of phenol were removed by washing the precipitate with 70% ethanol at 0°C and/or by reprecipitation, after drying the pellet and resuspending in NETS buffer.

This Kirby/phenol procedure, modified from Kirby (1965), extracts total nucleic acid and at pH 9.0 is efficient in the extraction of poly(A)$^+$RNA. It is important if the first interface is large to re-extract the organic phase and this interface as loss of poly(A)$^+$RNA may occur.

RNA was extracted from pooled sucrose gradient fractions by making up to 0.1 M NaCl, 10 mM EDTA, 0.5% SDS, 0.1 M Tris HCl pH 9.0

and extracting as above but with an equal volume of phenol: chloroform (1:1) instead of phenol/cresol mixture, to ensure that the aqueous phase was less dense.

(h) Oligo (dT)-cellulose affinity chromatography

Initial method

Columns of bed volume approximately 0.5 or 1 ml were formed in 2 ml plastic syringes and RNA samples dissolved in 0.5 ml binding buffer were loaded onto the pre-equilibriated columns with a flow rate of about 0.5-1.0 ml/min followed by 1 ml of binding buffer. The unbound (void) material was applied to the column once again followed by 2 x 2 ml of binding buffer to remove any unbound material. The bound material was eluted with 2-3 ml of elution buffer. Between samples columns were washed with and stored in 0.1 M NaOH.

Standard method

As explained in Chapter 3 improvements to this initial method were considered necessary and oligo (dT) bound RNA was isolated using the following more stringent conditions. The RNA sample, dissolved in 0.25-0.5 ml of binding buffer, was heated to 65°C for 2 minutes then chilled to room temperature and loaded onto the pre-equilibrated column using a slow flow rate (less than 0.5 ml/min), followed by an equal volume of binding buffer. This void material was then reloaded onto the column followed by an equal volume of binding buffer and the total void then reloaded a third time followed by 4 ml binding buffer. The bound fraction was eluted with 2 ml of elution buffer, made up to 0.4 M NaCl, heated to 65°C for 2 minutes, chilled and loaded onto a regenerated column, washed with binding

buffer and then finally eluted in 4 ml of elution buffer to give a bound-bound fraction. If the RNA in this fraction was to be precipitated it was necessary to add carrier RNA and to make at least 0.1 M NaCl before adding 2.5 volumes of ethanol at -20°C.

The yields of poly(A) in the void and bound-bound fractions were determined by poly(U) hybridization, where applicable, as described in section (k). Using stage 6 oocyte RNA 60-70% of the poly(A) was generally recovered in the bound-bound fractions compared with greater than 90% using previtellogenic ovary RNA.

(i) Sucrose gradient centrifugation

Analysis of RNA was performed using linear 7-30% sucrose gradients in NETS buffer. These were formed using a perspex gradient maker by placing light sucrose in the reservoir chamber and heavy sucrose in the mixing chamber and stirring with a spatula driven by an electric motor. The sucrose was pumped from the mixing chamber using a Perpex peristaltic pump and collected in the polycarbonate centrifuge tubes by pouring down the side. Gradients were usually poured the day before use and kept, covered at 30°C, until use.

RNA samples were dissolved in NETS buffer, heated to 65°C for 2 minutes and rapidly cooled before loading and the gradients were usually spun at 25°C. The rotors commonly used were the MSE 6 x 14 Ti, and the MSE 6 x 15 Al. The aluminium rotor gave highly satisfactory results, however a number of gradients were destroyed when using the titanium rotor because the caps developed hairline cracks and the subsequent evaporation was initially thought to be due to problems with the sealing rings. The centrifuges generally used were the MSE Superspeed 65 and 75, and only the former was problematic in that

on occasions the temperature control was faulty and the SDS precipitated during the run. The actual spinning times and speeds used are shown in the figure legends.

At the end of the run, a stainless steel needle was carefully inserted to the bottom of the gradient tube through which 40% sucrose was pumped (usually at 2 ml/min) into the tube and the gradient was displaced upwards through the duct in the perspex gradient plug and upwards through the flow cell of an ISCO 222 UV analyser and the absorbance at 254 nm was continuously recorded on a Bryans chart recorder. Usually 0.5 ml (15 sec) fractions were collected by hand, giving 24 fractions from a 12 ml gradient. These fractions were often frozen overnight before TCA precipitation and the recovery of radioactivity from the gradients was usually greater than 80%. Where lower recoveries were obtained this is recorded in the figure legends. In the figures the direction of centrifugation is from left to right, the last fraction being the pellet, and the actual absorbance trace, which was frequently due to carrier RNA is not usually given.

Discontinuous sucrose gradients were used to band mitochondria and were prepared by layering 2 ml of 1.4 M sucrose, 2.5 ml of 1.1 M sucrose and 3.5 ml of 0.8 M sucrose in 1 mM EDTA, 30 mM Tris HCl pH 7.4 at 2°C. Oocytes were very gently homogenized in ice cold 0.25 M sucrose, 1 mM EDTA, 30 mM Tris HCl pH 7.4 and centrifuged at 2,000 rpm for 7 minutes in the Sorval HB4 rotor at 0°C to pellet nuclei, yolk and membrane fragments. Up to 4 ml of the supernatant was loaded onto one of the above gradients and spun for 1 hour 15 minutes at 25,000 rpm at 4°C in the MSE 6 x 15 ml aluminium rotor with the brake off. At the end of the run the gradients, on ice, were pumped

out as described above and the mitochondria, which banded at the 1.1/1.4 M sucrose interface, were collected separately from the rest of the gradient. RNA was extracted from these gradient fractions as described in section (g) and the SDH activity determined as described in section (n).

Ribonuclease contamination of the sucrose solutions used for preparing sucrose gradients was prevented by pre-treatment of these solutions with DEP (20-100 µl per 100 ml of solution) which destroys proteins by attacking $-NH_2$ groups. Since DEP also reacts with RNA it was removed by heating the solution to 70°C for at least 1 hour which converts the DEP to ethanol and CO_2, and the pH was adjusted to 7.5 using Tris buffer.

(j) Digestion of RNA and preparation of poly(A)

Poly(A) was prepared from RNA samples by digestion with DNAse and RNAse T_1. A sample of RNA (up to 0.2 mg) was dissolved in 1 ml of a buffer containing 0.1 M NaCl, 1 mM $MgCl_2$, 10 mM Tris HCl, pH 7.0 and was treated with 0.04 mg/ml DNAse I (electrophoretically pure) for 30 minutes at 37°C. EDTA was added to 10 mM, followed by RNAse T_1 to give a RNA:RNAse T_1 ratio of 20:1 (w/w) and 2 µg RNAse A was also added. After a further 30 minutes at 37°C, the mixture was chilled on ice and precipitated immediately by adding 2.5 volumes of ethanol and 10-50 µg of E. coli RNA as carrier if the poly(A) was to be assayed by poly(U) hybridization. If the RNA sample was radioactive it was made 0.1% SDS and 0.4 M NaCl (i.e. oligo(dT) binding buffer), heated to 65°C for 5 minutes to destroy the DNAse, and then loaded onto oligo(dT)-cellulose to extract the radioactive poly(A) from resistant radioactive oligonucleotides prior to ethanol precipitation as above.

(k) ^{3}H-poly(U) binding assays

Poly(A) was estimated by measuring the amount of ^{3}H-poly(U) rendered ribonuclease resistant by annealing to the RNA as described by Bishop et al. (1974). The ^{3}H-poly(U) used in this investigation was prepared essentially as described by Bishop et al. (1974) and had a specific activity of 700,000 cpm/μg poly(A). The sample, dissolved in 1 ml 2 x SSC, was annealed with an excess of ^{3}H-poly(U) for 30 minutes at 45^{o}C, digested with 20 μg/ml RNAse A at 0^{o}C for 20 minutes and the nuclease resistant hybrids were precipitated by adding 0.5 ml of 4% CTAB and 0.5 ml of 1 M sodium acetate, pH 5.0 containing 2 mg/ml yeast RNA carrier. The precipitate was collected on Whatman GF/C filters, washed with 5-10 ml of water, dried and scintillation counted as described in section (m). Controls without RNA but with ^{3}H-poly(U) and RNAse, were subtracted from the sample counts. The specific activity of the ^{3}H-poly(U) was determined by annealing with known amounts of a standard poly(A) solution. Using these conditions the stoichiometry of the hybrids was 2 poly(U):1 poly(A) for all poly(A) sizes greater than $(A)_{10}$ (Darnbrough, personal communication).

(l) Polyacrylamide gel electrophoresis

RNA was analysed by acrylamide gel electrophoresis on cylindrical gels containing 2.3/0.115% acrylamide/bisacrylamide in gel buffer (36 mM Tris, 30 mM NaH_2PO_4, 2 mM EDTA pH 7.8). The gels were prerun at 7 mA/gel for 30 minutes and then samples, dissolved in 20-50 μl of gel buffer containing 0.2% SDS, 10% glycerol and bromophenol blue, were loaded onto the gels and electrophoresis was continued at 7 mA/gel until the dye was near the bottom of the

gel. Marker gels containing rRNA were usually run in parallel and gels with sufficient RNA present were scanned at 260 nm using a Gilson spectrophotometer with a gel scanning attachment. To determine radioactivity present, gels were sliced with a Mickle gel slicer and 0.8 or 1.0 mm slices were solubilized in scintillation vials by incubation overnight in the dark at 37°C in 4 ml of gel solvent (7.2 g PPO, 200 mg POPOP, 1800 ml toluene, 200 ml NCS and 18 ml 1% SDS). Using this method recovery of radioactivity from the gels wasusually greater than 80% of input.

Poly(A) was analysed on 9 cm cylindrical gels containing 10%/0.25% acrylamide/bisacrylamide in gel buffer. These gels were prerun for 30 minutes at 7 mA/gel and the poly(A) samples similarly dissolved in gel buffer containing 0.2% SDS, 10% glycerol and bromophenol blue before loading. Electrophoresis was performed at 7 mA/gel until the dye was approximately half way down the gel. Marker gels, with 4S RNA, 5S RNA and bromophenol blue were run in parallel and were scanned at 260 nm as for RNA gels. The sample gels were sliced with a Mickle gel slicer and two adjacent 1 mm slices were homogenized in 1 ml of 2 x SSC and were incubated for 30 minutes at 37°C to elute the poly(A) before spinning out the acrylamide. The pelleted acrylamide was washed with a further 0.5 ml of 2 x SSC and the poly(A) was thus recovered in a volume of 1.2 ml/fraction. If the poly(A) was radioactive it could be precipitated directly with CTAB or TCA, but if the poly(A) was to be assayed with ^{3}H-poly(U) (and this could also be performed on a small amount of radioactive poly(A)) then an excess of ^{3}H-poly(U) was added to each fraction and the ^{3}H-poly(U) binding determined as described in the preceeding section. The yield of poly(U) binding was normally in the range 85-110% of input.

Correction was made for poly(A) added in the E. coli RNA carrier, but was usually insignificant.

(m) Scintillation counting procedures

Samples of RNA and gradient fractions were usually precipitated on ice with TCA at a final TCA concentration of 5-10% for 20-30 minutes with 250 μg of BSA carrier. The precipitates were collected on Whatman GF/C filters washed twice with 5 ml of 5% TCA and once with 5 ml of ethanol and then dried in a vacuum oven at 80°C. The filters were then placed in plastic scintillation vials and counted using 2 ml of a scintillant containing 8 g/l butyl-PBD in toluene. CTAB precipitates from poly(U) binding assays were processed in the same manner. In later experiments plastic or glass inserts were used in the plastic vials but this resulted in little change in the counting efficiency which was 98% for ^{32}P and 24% for ^{3}H. Samples were counted on a Beckman L 200 Scintillation Counter or occasionally a Beckman LS 230 Scintillation Counter with a background of 20-30 cpm for ^{3}H and 5-10 cpm for ^{32}P which was always subtracted during data processing. In double label experiments the latter scintillation counter was always used and efficiencies and spillovers between channels were determined during each experiments using control TCA precipitated ^{3}H or ^{32}P labelled RNA.

(n) Succinate dehydrogenase assay

Succinate dehydrogenase (SDH) which is tightly bound to the outer mitochondrial membrane was assayed by its ability to convert the blue coloured oxidized form of the dye 2, 6 dichlorophenol-indophenol to its colourless reduced form, two hydrogen atoms being passed from the succinate ion, via the enzyme's FAD group, to the dye molecule. The decrease in absorbance at 600 nm by the oxidized dye

is proportional to the amount of enzyme present.

Reaction mix was made up by mixing 20 ml of distilled water and 10 ml of each of the following stock solutions: 0.3 M KH_2PO_4 pH 7.6; 6 mM KCN; 0.24 mM 2, 6 dichlorophenol-indophenol and 0.12 M sodium succinate. An aliquot of sample (oocyte homogenate or gradient fraction), made up to 0.2 ml was added to 1.0 ml of reaction mix and incubated at 37°C for 30 minutes to 1 hour or until the blue colour in some of the samples began to fade. The samples were then placed on ice and the tubes filtered through Whatman GF/C filters to remove suspended cell debris and the absorbance at 600 nm measured relative to control samples. The decrease in absorbance in duplicate samples were in good agreement and different dilutions of the same sample were proportional providing there had been sufficient change in absorbance.

(o) RNA polymerase assay

Before arriving at the optimum conditions for measuring the specific radioactivity of NTPs in PCA extracts from oocytes several slightly different methods were tried as explained in Chapter 6. In all the methods used the reaction mixture contained 1/5 volume (10 µl) of a stock assay mix (containing either one radiolabelled NTP, or all four NTPs only one of which was radiolabelled, in 0.25 M Tris HCl pH 7.9, 0.5 M $MgCl_2$, 1.0 M KCl, 0.09 M 2-mercaptoethanol), 10 µl of a DNA template, 5 µl of RNA polymerase core enzyme (approximately 2 units/µl in DNA buffer (see below) containing 50% glycerol. One unit of enzyme catalyzes the incorporation of 1 nmole of AMP in 1 hour under standard conditions (Berg et al., 1971)) and was made up to 50 µl with distilled water and/or PCA extract from the oocytes or unlabelled

NTP in order to construct calibration curves. An enzyme blank was always run as a control and usually the DNA was added just before the enzyme. The reaction mixture was incubated at 37°C for 20 minutes after which time 40 µl was removed onto a Whatman GF/C filter and washed immediately in 3 changes of ice cold 5% TCA containing 10 mM sodium pyrophosphate, followed by 2 changes of 70% ethanol. The filters were then dried and the radioactivity determined as described in section (m).

Method 1

In these experiments the stock assay mix contained 0.5 mM GTP, CTP and UTP and 2.0 mM ^{3}H-ATP at 0.25 x 10^{6} cpm/nmol in the above buffer. The DNA template was 2.5 mg/ml salmon sperm DNA in DNA buffer (10 mM NaCl, 1 mM EDTA, 10 mM Tris HCl pH 7.8). Up to 25 µl was available to add unlabelled ATP to construct a calibration curve.

Method 2

These experiments were carried out exactly as in method 1 except in the stock assay mix the concentration of GTP, CTP and UTP was increased from 0.5 mM to 9.0 mM.

Method 3

This is essentially the method of Maxson and Wu (1976) and the DNA template was poly [d(A-T)] (10 absorbance units/ml) in distilled water and up to 25 µl was available to add PCA extract from oocytes. To measure the specific radioactivity of ^{32}P-ATP in a PCA extract the stock assay mix contained 0.5 mM ^{3}H-UTP at 325,800 cpm/nmol in the

above buffer. To measure the specific radioactivity of ^{3}H-UTP the stock assay mix contained 1.6 mM ^{32}P-ATP at 150,700 cpm/nmol in the same buffer. In this double label method appropriate controls with only one radioisotope had to be done in order to measure the spillover between channels during scintillation counting.

(p) Basis for calculations

Number of poly(A) molecules

The number of poly(A) molecules is calculated from cpm of ^{3}H-poly(U) hybridized to gel eluates, by first subtracting the background cpm and then dividing by the specific activity of the ^{3}H-poly(U) (see section (k)) used in the experiment, giving the weight of poly(A) per gel slice. The number of AMP residues per poly(A) molecule in any slice was found from a calibration curve of the type obtained by Cabada et al. (1977) in which the ^{3}H-adenosine ratio in gel slices was determined by paper electrophoresis at pH 3.5. Dividing the weight of poly(A) per slice by the appropriate number of AMP residues in that slice and by the molecular weight of AMP gives the number of moles of poly(A) in that slice and this is easily converted to the number of molecules by multiplying by Avagadro's number.

A similar calculation can be performed in experiments where the poly(A) is labelled if the specific activity of the labelled poly(A) is known.

Rates of RNA synthesis

In discussions of the various rates of RNA synthesis in Chapter 7 weights of RNA synthesized have been calculated from pmoles NTP incorporated by assuming that for each pmole of NTP incorporated 1280 pg of RNA is synthesized (i.e. 25% base composition and an average molecular weight of 320 daltons for the NMP). This has been performed because base composition data is not available for all the RNA classes studied.

CHAPTER 3

RNA Synthesis in Stage 6 Oocytes Incubated In Vitro

(a) Introduction

In Chapter 1 the importance of studying RNA synthesis and in particular mRNA synthesis, during oogenesis has been discussed. As noted there, LaMarca et al. (1973) suggested that much of the earlier work on oogenesis in Xenopus laevis implied that stage 6 oocytes are relatively quiescent in RNA synthesis, however, these authors report that similar rates of RNA synthesis occur in stage 6 and stage 3 (maximal lampbrush stage) Xenopus laevis oocytes and most of the RNA made appears to be rRNA. No measurement of mRNA synthesis was made.

It was considered a useful starting point in this study of RNA synthesis during oogenesis in Xenopus laevis to try to verify the work of LaMarca et al. (1973) with respect to stage 6 oocytes and further to try to obtain information concerning mRNA synthesis in these oocytes. Since most eucaryotic mRNA molecules have a sequence of poly(A) at their 3' ends (Kates, 1970; Edmonds et al., 1971; Greenberg, 1975), it was reasoned that if rapid RNA synthesis was shown to occur in stage 6 oocytes attempts should be made to isolate a fraction of the newly labelled RNA on oligo (dT)-cellulose.

Of necessity some preliminary experiments had to be performed to establish efficient techniques of oocyte manipulation and these are described in the first part of this Chapter.

Further, since considerable evidence suggests that much of the messenger RNA synthesized during oogenesis is made during the lampbrush chromosome stage and enters a stored pool of transcripts (Davidson, 1968, 1976; Rosbash & Ford, 1974), it was considered useful to try to discover the cellular localization and site of synthesis of any newly made RNA in stage 6 oocytes, by using the

germinal vesicle isolation technique of Gall (1966). The second part of this Chapter describes experiments of this type. The final part of Chapter 3 reports experiments designed to improve some of the techniques which were found to be unsatisfactory.

(b) Development of conditions for oocyte incubations

A number of experiments were performed in order to develop suitable conditions for the incubation of oocytes in vitro. Typically the procedure was as follows.

Stage 6 oocytes were prepared from ovary in the ways detailed in Table 3-1 and Table 3-2. The freed oocytes were then incubated in radioactive guanosine for 20 hours, after which time they were further treated as described in Tables 3-1 and 3-2 and then homogenized in Kirby's medium and the RNA extracted as described in materials and methods section (g). Aliquots of the RNA were CTAB precipitated to determine the cpm of radioactive guanosine incorporated into the oocytes under the various conditions.

From these tables it is clear that most samples show a good level of guanosine incorporation. Despite the variation, it is however obvious that a substantial proportion of the incorporation is due to the follicle cells. In Table 3-2 where the follicle cells have been recovered about 60% of the observed incorporation is due to the follicle cells.

The use of pronase or collagenase alone to remove follicle cells was shown, by microscopic examination of the treated oocytes, not to be completely successful. Manual removal of the follicle cells using watchmakers forceps was on the other hand completely successful as judged by this method (Colman, 1974). Therefore some of the

Table 3-1 The effect of manipulations on uptake and incorporation of 8-^{3}H-guanosine by stage 6 Xenopus laevis oocytes

Oocyte Manipulations	A	B	C	D
before incubation	oocytes manually stripped from ovary			oocytes freed from ovary with collagenase
after incubation	-	follicle sheaths removed with pronase	follicle sheaths removed with collagenase	
Resulting sample	Control oocytes and follicle sheaths	oocytes with follicle sheaths partially removed		
Sample 1				
Uptake	20,017	19,280	21,027	6,552
Incorporation	648	602	577	93
Sample 2				
Uptake	18,788	11,924	19,914	5,280
Incorporation	596	567	674	47
Mean				
Uptake	19,403	15,602	20,471	5,916
Incorporation	622	585	626	70

Duplicate batches of 20 stage 6 oocytes were prepared from fresh ovary by either manual stripping with watchmakers forceps (columns A, B and C) or by incubation in MBX containing collagenase (2 mg/ml) and 20 mM EDTA for 30 minutes (column D). The isolated oocytes were then washed with MBX and incubated for 20 hours in 50 μl of MBX containing 8-^{3}H-guanosine (100 μCi/ml). After incubation the oocytes were washed several times and either homogenized immediately in Kirby buffer (columns A and D) or first treated for 15 minutes at 20°C with MBX containing 20 mM EDTA and 2 mg/ml of either pronase (column B) or collagenase (column C). Samples of the total homogenate were dried onto GF/C filters and the radioactivity determined as described in materials and methods section (m). This is a measurement of the uptake of guanosine and the data is expressed as cpm/oocyte. RNA was extracted from the rest of the homogenate (materials and methods section (g) and samples of total RNA were precipitated with CTAB and the radioactivity determined. This is a measure of the incorporation and is expressed as cpm/oocyte. In all samples approximately 4.4 μg RNA/oocyte was recovered.

Table 3-2 The effect of oocyte manipulations on uptake and incorporation of 8-^{3}H-guanosine by fresh and 2 day old stage 6 Xenopus laevis oocytes

The experimental details are similar to those of Table 3-1, in this case using both fresh and 2 day old oocytes, except that the batches of oocytes were incubated in 100 µl of MBX containing 8-^{3}H-guanosine (100 µCi/ml). After incubation the follicle sheaths were manually dissected off the oocytes after they had been incubated for15 minutes in MBX containing collagenase (2 mg/ml) and 20 mM EDTA (Column B). This collagenase treatment was performed before incubation for the oocytes in column D. Column E oocytes were exposed to collagenase treatment before incubation which only partially removed the follicle sheaths. The labelled follicle sheaths from (B) were also homogenized and extracted. Measurements of uptake and incorporation were made as described in Table 3-1 and the data is similarly expressed.

Table 3-2 The effect of oocyte manipulations on uptake and incorporation of 8-^{3}H-guanosine by fresh and 2 day old stage 6 Xenopus laevis oocytes

Oocyte manipulations	A	B	C	D	E
before incubation	Oocytes manually stripped from ovary			oocytes stripped then follicles removed manually with collagenase	oocytes stripped then follicles removed with collagenase only
after incubation	-	follicles removed with collagenase manually	-	-	-
resulting sample	control oocytes and follicle sheaths	oocytes	follicle sheaths from (B)	oocytes	oocytes with follicle sheaths partially removed
Fresh Ovary:					
Sample 1					
Uptake	20,689	14,944	5,445	11,802	5,698
Incorporation	248	100	153	89	111
Sample 2					
Uptake	19,134	18,512	3,899	12,922	3,705
Incorporation	246	120	145	104	37
Mean					
Uptake	19,912	16,728	4,672	12,326	4,701
Incorporation	247	110	149	97	74
2 Day Old Ovary:					
Sample 1					
Uptake	26,611	25,541	4,420	11,585	3,997
Incorporation	309	49	184	86	61
Sample 2					
Uptake	24,808	19,628	6,578	15,845	3,997
Incorporation	289	71	221	240	197
Mean					
Uptake	25,710	22,585	5,499	13,718	3,997
Incorporation	298	60	203	162	129

As a result of the experiments outlined above it was decided to adopt the following conditions of incubation for future experiments.

Where possible oocytes should be prepared from an ovary which was recently removed from the frog, the oocytes being manually stripped from the ovary by careful use of watchmakers forceps. Oocytes should be incubated for the desired time with their follicle sheaths present and at the end of the incubation the oocytes must have their follicle sheaths removed manually. If no further incubation is to be undergone by the oocytes, collagenase and in certain circumstances shrinking medium can be used to aid the manual removal of follicle sheaths from oocytes especially when this has to be done rapidly. Similar conclusions concerning the conditions of oocyte incubations have recently been published (Colman, 1974).

(c) Kinetics of uptake and incorporation of nucleosides in incubated stage 6 oocytes and their follicle cells

Initial attempts to study the kinetics of RNA synthesis in stage 6 oocytes involved incubating the oocytes in 8-^{3}H-guanosine or 2-^{3}H-adenosine for various times as detailed in materials and methods section (c). At the end of the experiment the follicle sheaths were dissected off the oocytes and the oocytes and follicle sheaths were homogenized separately in modified Kirby buffer and the RNA extracted. A sample taken from the total homogenate was counted directly and this is a measure of the uptake of nucleoside from the medium. Samples of extracted nucleic acid were precipitated with TCA to determine the incorporation.

Uptake

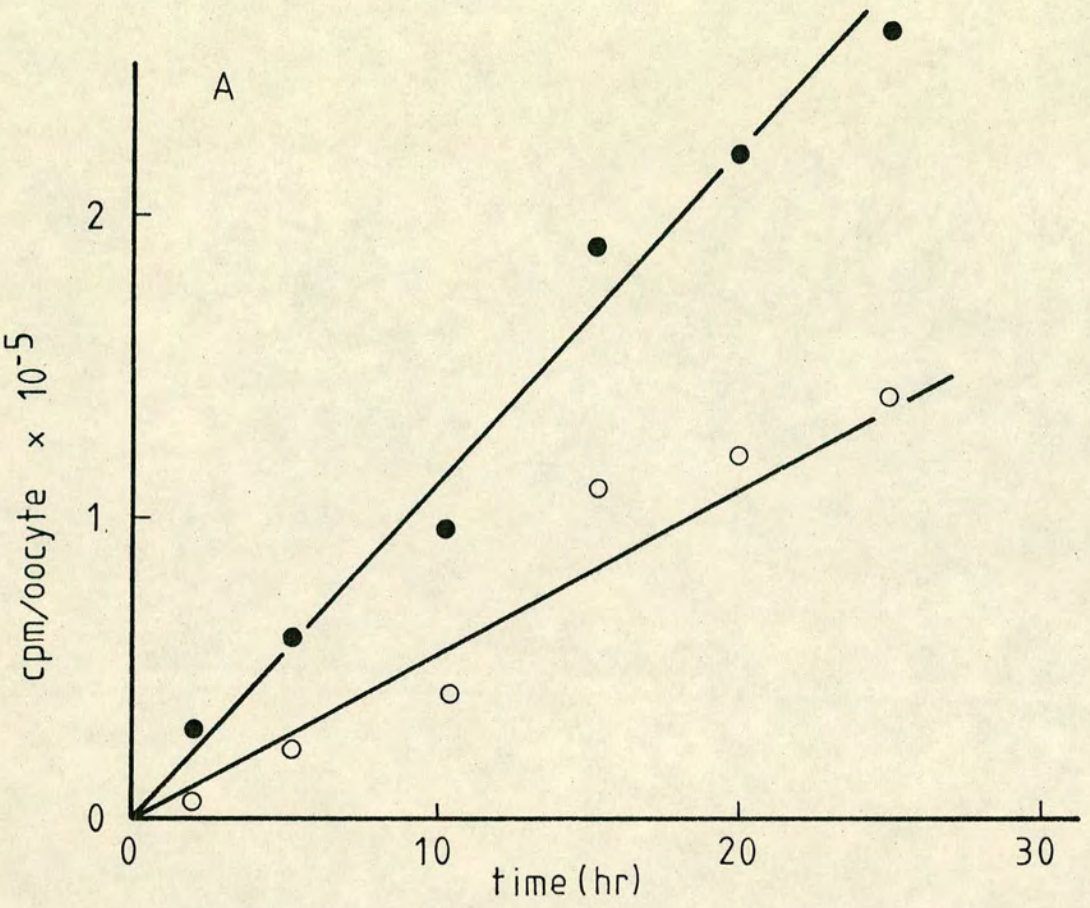
Figure 3-1A shows the uptake of 2-^{3}H-adenosine and 8-^{3}H-guanosine by stage 6 oocytes from the same female. Consistent with other work (LaMarca et al., 1973; Colman, 1974), at least up to 30 hours the uptake of both these nucleosides is approximately linear. This suggests that the oocytes remain healthy during the course of the experiments and that the amount of nucleoside in the medium does not become limiting. Adenosine enters the oocytes faster than guanosine and since guanosine enters stage 6 Xenopus oocytes faster than uridine (Colman, 1974) then if the oocyte nucleoside pool sizes are in proportion to the NTP pool sizes measured by Woodland and Pestell (1972), the rate of uptake of nucleosides would not appear to be related to the intracellular pool size. Figure 3-1B shows the uptake for the follicle sheaths. In this case uptake levels off within 10 hours of incubation for both nucleosides.

In oocytes, if the nucleoside and NTP pools are in equilibrium, it might be expected that the NTP specific radioactivity will be increasing linearly since the uptake of nucleoside is linear and this has been shown to be the case (LaMarca et al., 1973). For follicle cells on the other hand the same reasoning would predict a constant NTP specific radioactivity after about 10 hours of incubation.

In these experiments the choice of radioactive precursor is dependent on a number of factors.

(1) Radioactive guanosine has been used as a specific label for RNA synthesis in stage 6 oocytes, since DNA synthesis is extremely low (Gurdon, 1967; Mairy & Denis, 1971).

(2) Uridine entry into stage 6 Xenopus laevis oocytes is slower than for guanosine (Colman, 1974) and therefore RNA of higher


A
cpm/oocyte x 10-5
0
1
2
0
10
20
30
time (hr)

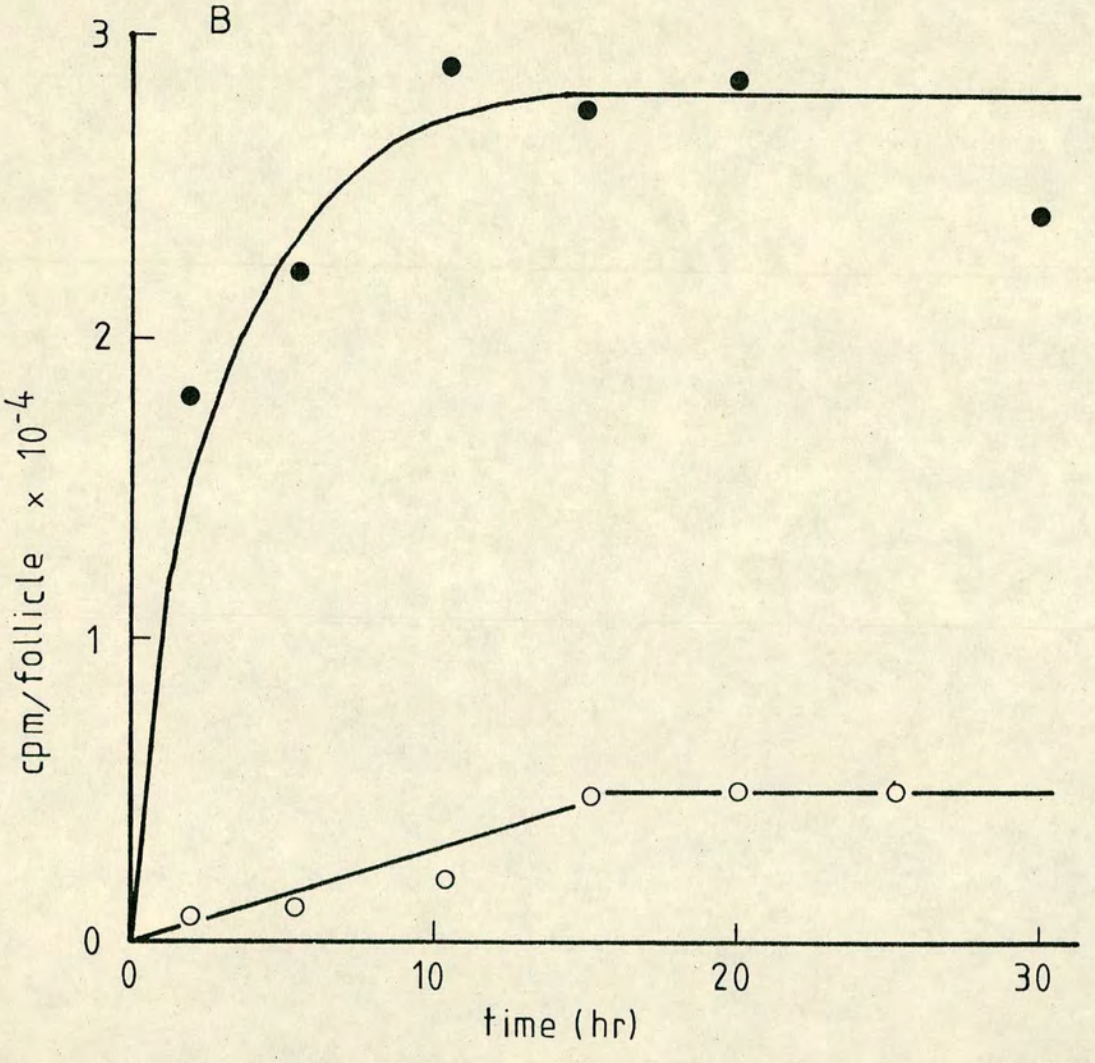

B
cpm/follicle x 10-4
0
1
2
3
0
10
20
30
time (hr)

Figure 3-1 In vitro uptake of ^{3}H-nucleoside by stage 6 oocytes and follicle sheaths of Xenopus laevis

About 1,400 stage 6 oocytes were manually stripped from an ovary and incubated in batches of 30 oocytes in 60 µl of MBX containing 1 mCi/ml ^{3}H-nucleoside. At the time points indicated the oocytes were washed 3 times in MBX and then the follicle sheaths were rapidly removed as described in materials and methods section (e). For each time point 4 batches of 30 oocytes were processed and for the early time points it was necessary to stagger the incubations since follicle removal took between 15 and 30 minutes. The oocytes and follicle sheaths were counted and collected separately in 4 ml of modified Kirby buffer and homogenized, 100 µg of total ovary carrier RNA being added to the follicle sheath samples. Aliquots (50 µl) of homogenate were placed directly onto GF/C filters, dried and the radioactivity determined as described in section (m) of materials and methods and this determination represents the uptake of nucleoside from the medium. Correction was made for the quenching effect of modified Kirby buffer. RNA was extracted from the remaining homogenate as outlined in materials and methods section (g).

(A) Uptake of ^{3}H-nucleoside by stage 6 oocytes.

(●) Uptake of ^{3}H-adenosine,
(O) uptake of ^{3}H-guanosine.

(B) Uptake of ^{3}H-nucleoside by follicle sheaths.

(●) Uptake of ^{3}H-adenosine,
(O) uptake of ^{3}H-guanosine

Data expressed as cpm of ^{3}H-nucleoside per oocyte or follicle sheath.

specific radioactivity will result using radioactive guanosine.

(3) Radioactive cytidine is not a good label with which to study RNA synthesis in the absence of DNA synthesis since incorporation into the -CCA terminus of tRNA molecules will occur.

(4) Although objection (3) is also applicable when using radioactive adenosine to label RNA, if one is interested in labelling poly(A)$^+$RNA to the highest specific activity, or in labelling the poly(A) itself, there is no alternative but to use radioactive adenosine.

In the experiments which follow 2-^{3}H-adenosine or 8-^{3}H-guanosine have been used to label the RNA.

Incorporation into RNA

The kinetic curves of incorporation of adenosine and guanosine into TCA precipitable material by stage 6 oocytes incubated in vitro are given in Figure 3-2A, and the results show a significant deviation from linear incorporation up to 30 hours. At early times the data points are best fitted to a parabolic curve since the specific activity (SA) of the NTP precursor pool increases linearly with respect to time (t) at least up to 30 hours (LaMarca et al., 1973).

$$SA = kt$$

The observed rate of incorporation (dI/dt) of NTP into stable RNA will be determined by the net rate of synthesis of stable RNA (R) and by the NTP specific activity.

$$dI/dt = R \times SA$$

Therefore, by substituting and integrating,

$$I = \tfrac{1}{2}Rkt^2 + C$$

and since at zero time there can be no incorporation.

$$I = \tfrac{1}{2}Rkt^2$$

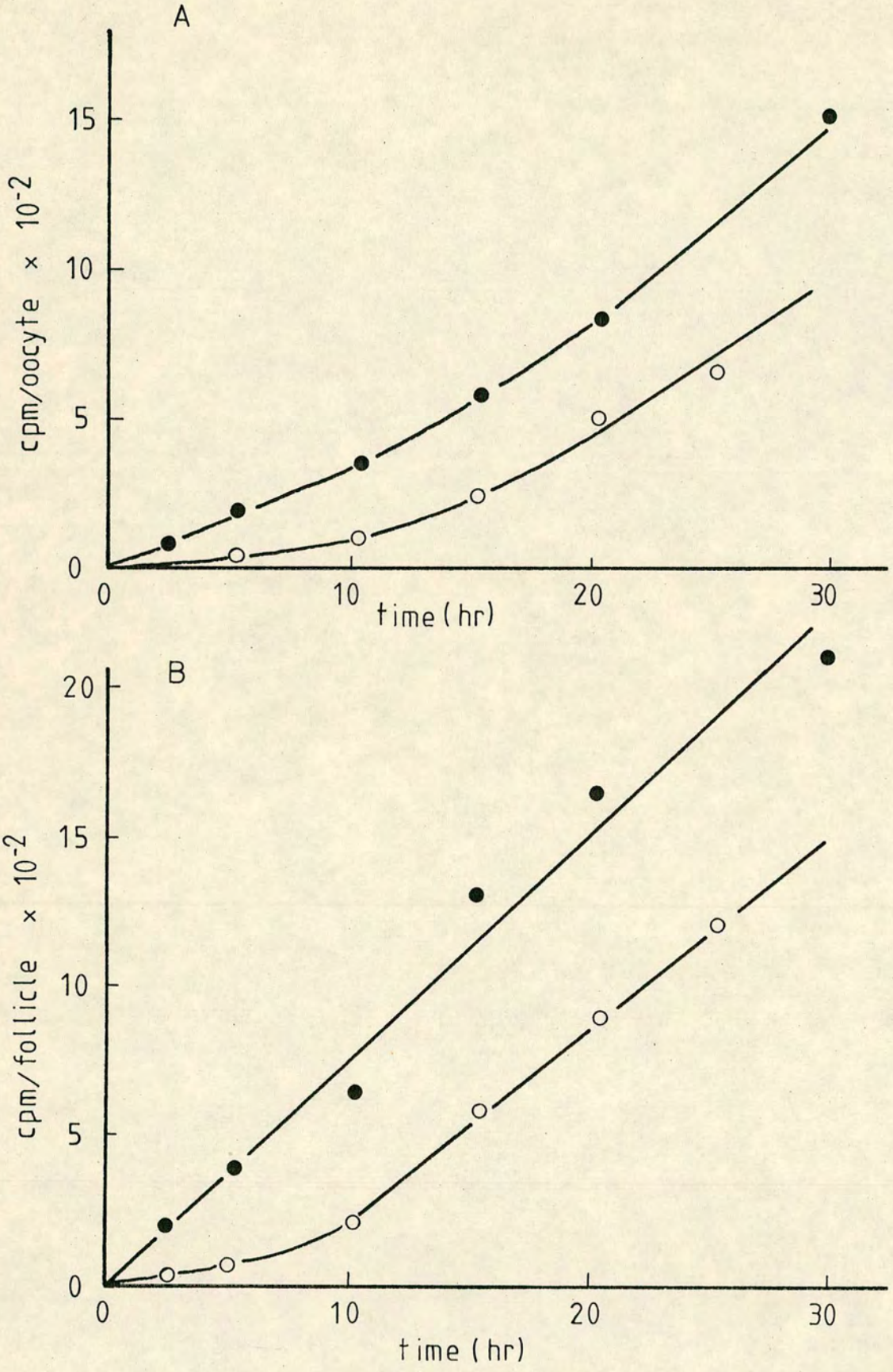

A
15
10
5
0
cpm/oocyte × 10-2
0
10
20
30
time (hr)
B
20
15
10
5
0
cpm/follicle × 10-2
0
10
20
30
time (hr)

Figure 3-2 Kinetics of incorporation of ^{3}H-nucleoside into RNA in stage 6 oocytes and follicle sheaths of Xenopus laevis incubated in vitro

Samples of total RNA prepared in the experiment described in Figure 3-1 were precipitated with TCA and the radioactivity determined as described in materials and methods section (m). Data expressed as TCA precipitable cpm incorporated per oocyte or follicle sheath.

(A) Incorporation of ^{3}H-nucleoside into stage 6 oocyte total RNA.

(●) incorporation of ^{3}H-adenosine,
(O) incorporation of ^{3}H-guanosine.

(B) Incorporation of ^{3}H-nucleoside into follicle sheath total RNA.

(●) incorporation of ^{3}H-adenosine,
(O) incorporation of ^{3}H-guanosine.

Therefore providing the specific activity of the precursor pool of NTP increases linearly the observed rate of incorporation into stable RNA will vary directly with the square of the time and there will be no intercept. The data in Figure 3-2A for both adenosine and guanosine is consistent with this interpretation and is also in agreement with LaMarca et al. (1973) who studied incorporation of guanosine *in vitro* into *Xenopus laevis* oocytes over 15 hours.

At longer incubation times this conclusion may not hold. My own results and those of other workers (Colman, 1974) suggest that the net uptake of nucleosides by stage 6 *Xenopus laevis* oocytes incubated *in vitro* ceases after about 2-3 days. When this occurs the specific activity of the NTP precursor should be constant and therefore the observed incorporation into RNA should become linear with respect to time. Therefore, long incubations of stage 6 oocytes (4-5 days) should produce time courses of stable RNA synthesis which are initially parabolic but which become linear at some point after about 2 days.

The time courses of incorporation of radioactive adenosine and guanosine into RNA by the follicle sheaths of stage 6 oocytes incubated *in vitro* are given in Figure 3-2B. Although the data is not as good as for the oocytes it shows that incorporation of these nucleosides by follicle sheaths is approximately linear. This result would be expected if the specific activity of the precursor NTP was constant during the time course and if the nucleic acid synthesised was stable over this period. The measurements of uptake by the follicle sheaths described above show that saturation occurs relatively rapidly and there is therefore likely to be a constant

NTP precursor specific activity from about 5-10 hours. From the arguments outlined above for oocytes, the observed incorporation curve for follicles might be expected to be parabolic until the NTP specific activity becomes constant. The data for guanosine is not inconsistent with this hypothesis.

Analysis of the nucleic acid synthesized

Since the nucleosides used in these experiments may label DNA as well as RNA and the extraction procedure used (materials and methods section (g)) extracts total nucleic acid then the question arises is any of the observed incorporation by stage 6 oocytes due to DNA synthesis? The effect of NaOH hydrolysis of the TCA precipitable material extracted from stage 6 oocytes and their follicle sheaths is given in Table 3-3. It is clear that over 99% of the material labelled by radioactive adenosine in oocytes is alkali sensitive. This is consistent with the observation that DNA synthesis is absent in stage 6 oocytes (Gurdon, 1967).

Analysis of the nucleic acid synthesized during these incubations by sedimentation on sucrose gradients shows that the majority of the nucleoside incorporated is into various species of RNA. Sucrose gradient profiles of newly synthesized total RNA from stage 6 oocytes are given in Figure 3-3. The following observations can be made.

After 2 hours of incubation three peaks of radioactivity are visible. Near the top of the gradient 4S and 5S RNA, which do not separate under these conditions, account for about a third of the total incorporation. Other workers have attributed much of this low molecular weight RNA synthesis to 4S RNA (LaMarca et al., 1973; Colman, 1974). Near the bottom of the gradient, in the 40S

Table 3-3 Alkaline hydrolysis of 2-^{3}H-adenosine labelled nucleic acid

Sample	NaOH- Total TCA ppt. cpm	NaOH+ Total TCA ppt. cpm	% cpm remaining after NaOH
10 hr oocytes	83,770	472	0.56%
10 hr follicles	5,820	34	0.58%
20 hr oocytes	37,520	194	0.51%
20 hr follicles	14,112	122	0.86%
30 hr oocytes	84,616	585	0.69%
30 hr follicles	25,592	327	1.28%

Identical samples of nucleic acid extracted from stage 6 oocytes incubated in 1.0 mCi/ml 2-^{3}H-adenosine for 10, 20 and 30 hours, were incubated for 1 hour at 37^{o}C in the presence or absence of 2 M NaOH. The radioactivity remaining was precipitated with TCA as described in materials and methods section (m).

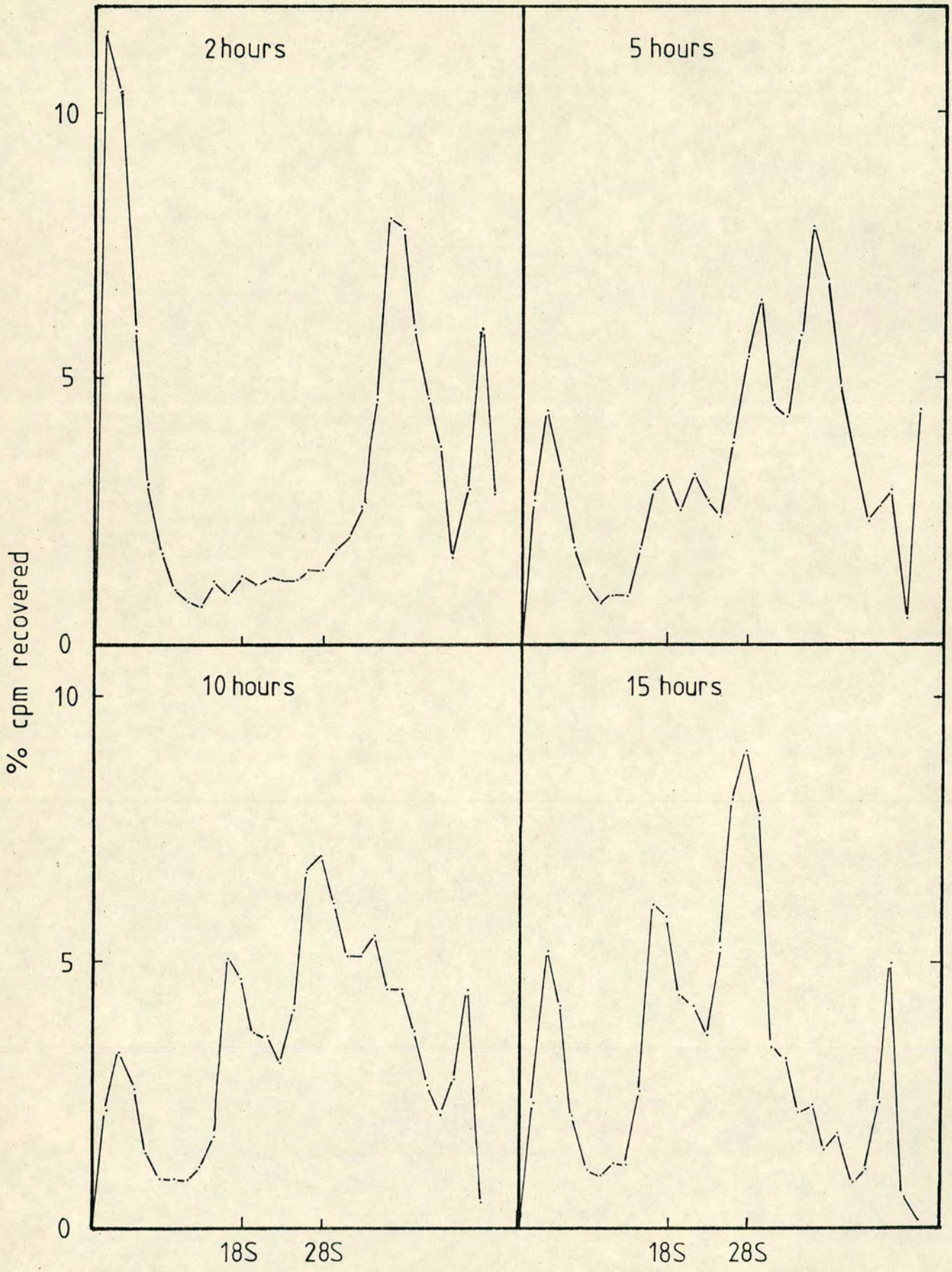

2 hours
5 hours
10 hours
15 hours
10
5
0
% cpm recovered
18S
28S
18S
28S

Figure 3-3 Sucrose gradient analysis of *in vitro* labelled total RNA from stage 6 oocytes of *Xenopus laevis*

Samples of oocyte total RNA which were labelled by incubation with ^{3}H-adenosine in the experiment described in Figure 3-1 were dissolved in 0.25 ml NETS and sedimented in 11.5 ml linear 7-30% sucrose gradients in NETS buffer at 40,000 rpm for 4½ hours at 25°C in an MSE 6 x 14 Ti rotor. The gradients were analysed and the radioactivity determined as described in materials and methods section (i). Recovery of loaded cpm was 70-80% and the figures show the percentage of the total recovered ^{3}H cpm which is present in each fraction. Centrifugation was from left to right and the last two fractions correspond to the pellet. The positions of 18S and 28S RNA are marked. The following cpm were recovered:

2 hours 2,150 cpm; 5 hours 9,700 cpm;
10 hours 25,600 cpm; 20 hours 26,100 cpm.

region, a peak of radioactivity is visible which also accounts for about a third of the total incorporation. This is the rRNA precursor (Loening et al., 1969; LaMarca et al., 1973). The third peak is radioactivity pelleting during the run (> 45S). At 2 hours no 18S or 28S rRNA is visible.

After 5 hours of incubation small peaks of radioactivity sediment at the positions of the 18S and 28S rRNA markers and also at about 24S. This latter species has been reported previously but its function is not known (Mairy & Denis, 1971; LaMarca et al., 1973). The percentage of incorporation due to 4S/5S RNA and to the rRNA precursor have both decreased. Synthesis and maturation of 18S and 28S rRNA molecules therefore takes between 2 and 5 hours in stage 6 oocytes incubated in vitro. Since there is little change in the absolute incorporation into the 40S rRNA precursor between 2 and 5 hours, it is likely that the half-life of this species is less than 2 hours, which is consistent with the data of Anderson & Smith (1977).

There is at all time points a large proportion of non-ribosomal RNA which sediments in a heterodisperse manner throughout the whole length of the gradients and which even after 15 hours could account for up to 50% of the total incorporation. Other investigators have made this observation (Anderson & Smith, 1977).

Figure 3-4 gives sucrose gradient profiles of total RNA from the stage 6 oocyte follicle sheaths. Comparison of Figure 3-3 with 3-4 show that similar gradient profiles are obtained for both oocytes and follicle sheaths and hence the conclusions listed above for stage 6 oocytes also apply in general to the synthesis of classes of RNA in follicle sheaths with the following exceptions.

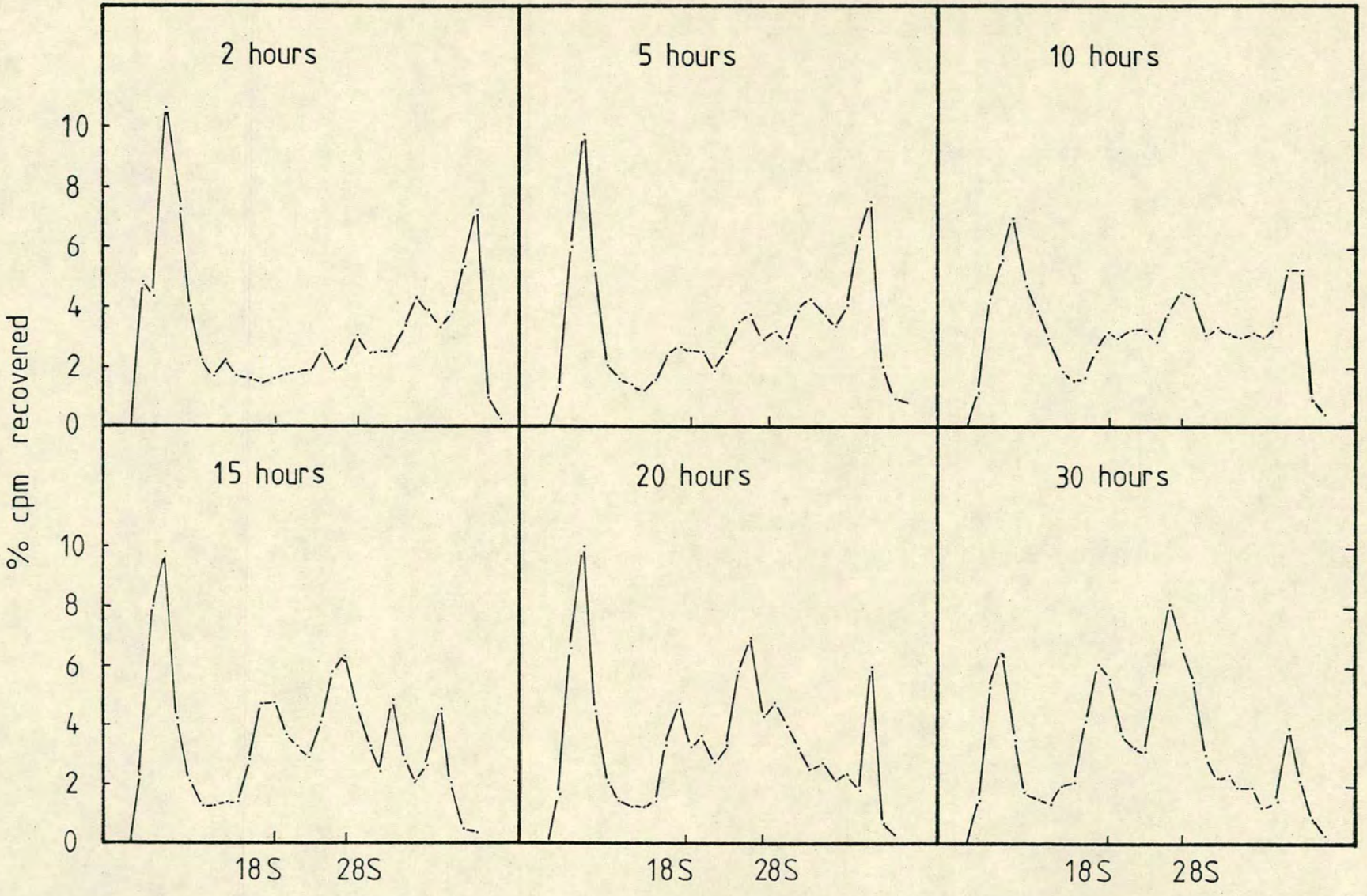

2 hours
5 hours
10 hours
15 hours
20 hours
30 hours
% cpm recovered
0
2
4
6
8
10
18S
28S

Figure 3-4 Sucrose gradient analysis of *in vitro* labelled total RNA from follicle sheaths

Samples of follicle sheath total RNA labelled with ^{3}H-adenosine from the experiment described in Figure 3-1 were sedimented in sucrose gradients exactly as described in Figure 3-3 for oocyte total RNA and the radioactivity was determined as described in section (m) of materials and methods. Recovery of loaded cpm was 70-80% and the figures show the percentage of the total recovered ^{3}H cpm which is in each fraction. The direction of centrifugation and the positions of the pellet are the same as in Figure 3-3. The following cpm were recovered:

2 hours 3,550 cpm; 5 hours 12,900 cpm; 10 hours 25,700 cpm;
15 hours 60,800 cpm; 20 hours 55,900 cpm; 30 hours 62,100 cpm.

The peak for 40S rRNA precursor in follicle sheaths is not so prominent as in oocytes and the amount of 4S/5S RNA synthesized as a percentage of the total RNA synthesized is significantly greater in follicle sheaths than in oocytes. If stage 6 oocytes synthesize little 5S RNA (LaMarca et al., 1973; Colman, 1974) this difference may be due to 5S RNA synthesis in the follicle sheaths. These observations suggest that the pattern of RNA synthesis in follicle cells is more like that of somatic cells in general than stage 6 oocytes which synthesize large amounts of 18S and 28S rRNA.

Oligo(dT) bound RNA

In order to discover whether it was possible to detect mRNA synthesis in stage 6 oocytes experiments were performed in which newly labelled total RNA was fractionated by hybridization to oligo (dT)-cellulose. In this procedure RNA which binds to the oligo (dT)-cellulose (oligo (dT) bound RNA) must contain poly(A) while RNA with little or no poly(A) (oligo (dT) void RNA) does not.

The time courses of incorporation of radioactive adenosine into oligo (dT) bound RNA in stage 6 oocytes and follicle sheaths are given in Figure 3-5. The oocyte curve is distinctly S-shaped, being parabolic to about 15 hours and then tending to level off. The simplest explanation for this could be that a class of oligo (dT) bound RNA with an average half-life of about 10 hours is being made under conditions where the specific activity of the NTP precursor is increasing linearly. However, this type of curve could also be generated by a mixture of stable and unstable components and the interpretation would be further complicated if the poly(A) itself turned over. The amount of incorporation of radioactive adenosine

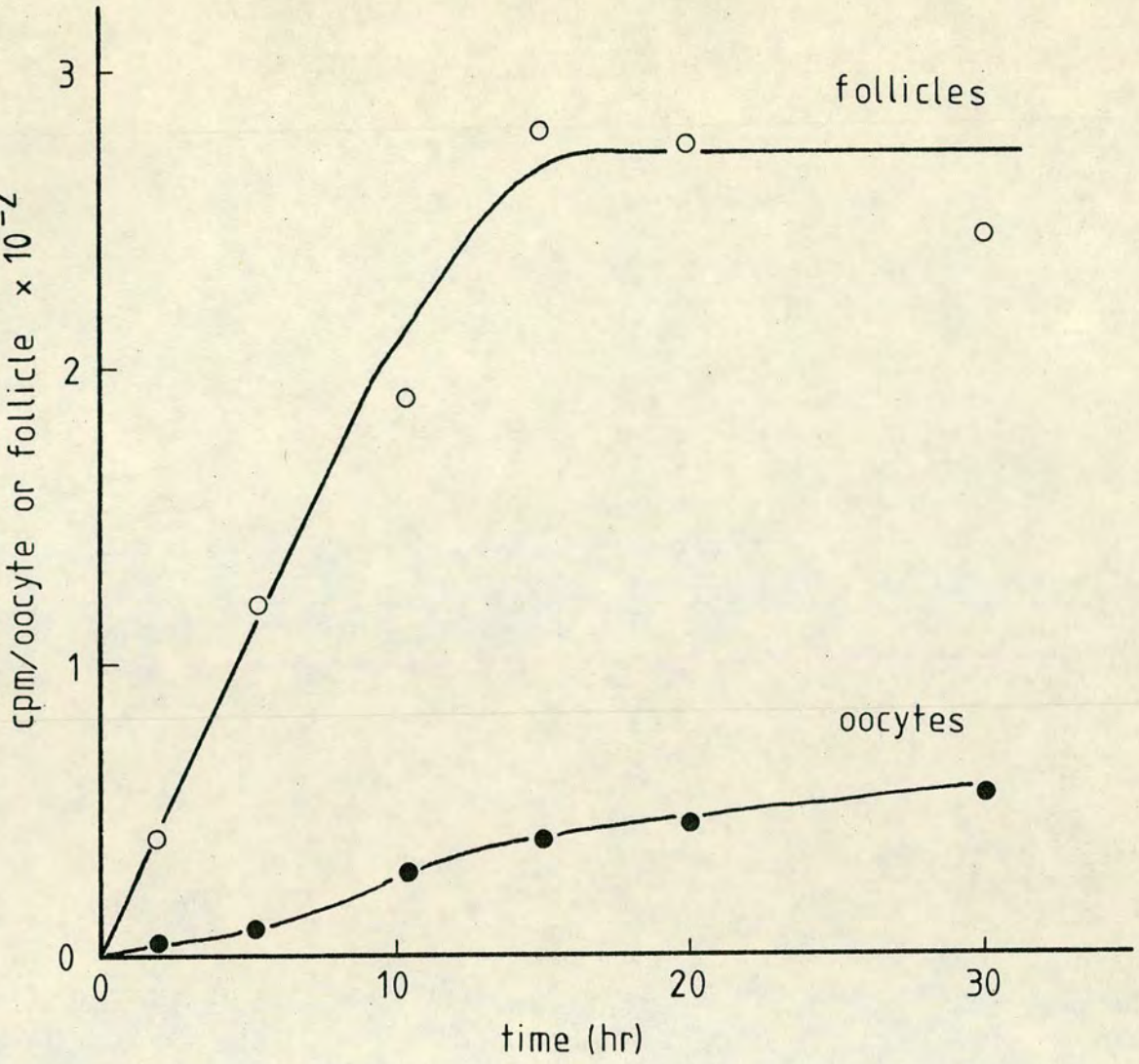


Figure 3-5 Kinetics of incorporation of ^{3}H-adenosine into oligo (dT) bound RNA in stage 6 oocytes and follicle sheaths of Xenopus laevis

Oligo (dT) bound RNA was prepared from the samples of total RNA, which were labelled by incubation with ^{3}H-adenosine in the experiment described in Figure 3-1, by using the method outlined in section (h) of materials and methods. Samples of the oligo (dT) bound RNA were precipitated with TCA and the radioactivity determined as described in materials and methods section (m). Recovery of loaded cpm after oligo (dT)-cellulose chromatography was over 98%. The data is expressed as TCA precipitable cpm per oocyte or follicle sheath.

(●) ^{3}H-adenosine incorporation into oocyte oligo (dT) bound RNA.

(O) ^{3}H-adenosine incorporation into follicle sheath oligo(dT) bound RNA.

into oligo (dT) bound RNA in preliminary experiments was within the range 2-8% of the incorporation into total RNA.

In Figure 3-5 the curve for the follicle sheaths shows a definite plateau and the simplest explanation is that the oligo (dT) bound RNA made in the follicle sheaths has a single half-life of about 6 hours. For follicle sheaths the amount of incorporation into oligo (dT) bound RNA was between 11 and 25% of the incorporation into total RNA. The 3 to 5 fold higher incorporation of adenosine into oligo (dT) bound RNA in follicle sheaths relative to oocytes must be due to a greater rate of oligo (dT) bound RNA synthesis in follicle cells since incorporation of radioactive adenosine into total RNA in this experiment was roughly equal for the two cell types.

In any attempt to describe accurately the kinetics of synthesis of different classes of RNA attention must be paid to the purity of the RNA in consideration. Therefore samples of oligo (dT) bound RNA were run on sucrose gradients and typical results are given in Figure 3-6. The newly synthesised oligo (dT) bound RNA in both oocytes and follicle sheaths shows a heterogeneous size distribution with a mean size around 18S. These gradient profiles are in reasonable agreement with the previously reported size distribution of stored poly(A)$^{+}$RNA in oocytes (Rosbash & Ford, 1974). From sample to sample the degree of contamination by rRNA species varied considerably and was often as high as 50%. This was particularly true at late time points where the percentage of incorporation into oligo (dT) bound RNA was as little as 2% of the incorporation into total RNA. It is clear that systematic variations in contamination such as this could lead to erroneous conclusions about the kinetics of synthesis of oligo (dT) bound RNA and therefore attempts were made to overcome this problem and are given later.

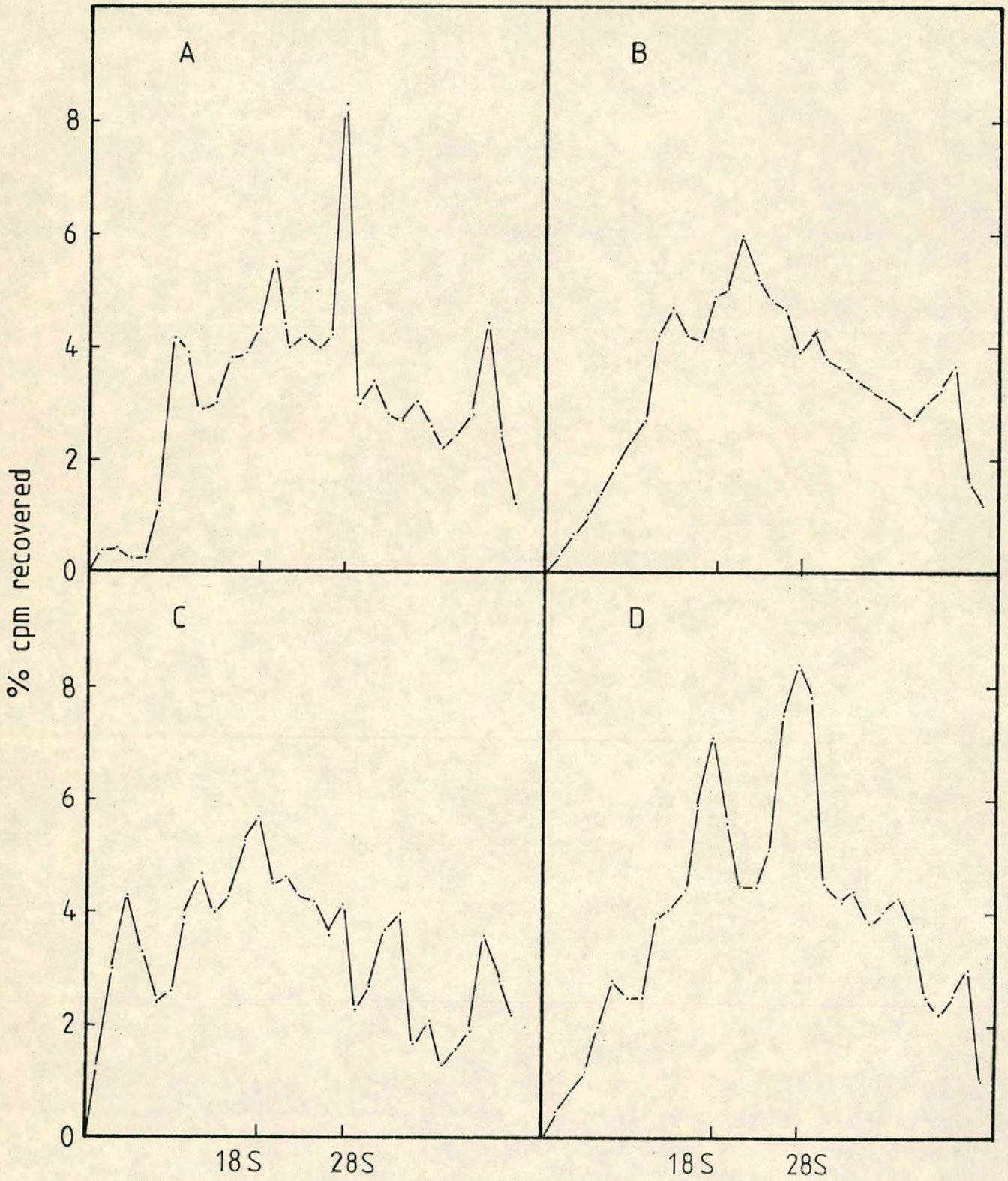

A
B
C
D
% cpm recovered
0
2
4
6
8
18S
28S
18S
28S

Figure 3-6 Sucrose gradient analysis of *in vitro* labelled oligo (dT) bound RNA from stage 6 oocytes and follicle sheaths of *Xenopus laevis*

Samples of oligo (dT) bound RNA prepared as described in Figure 3-5 were sedimented in 11.5 ml linear 7-30% sucrose gradients exactly as described in Figure 3-3. The gradients were analysed as outlined in section (i) of materials and methods and over 70% of the loaded cpm were recovered. The direction of centrifugation was from left to right and the last 3 fractions correspond to the pellet. The positions of the 18S and 28S carrier RNA are marked. The data is expressed as the percentage per fraction of the total cpm recovered.

A and B Follicle sheath oligo (dT) bound RNA labelled with ^{3}H-adenosine

A = 10 hours 4,650 cpm recovered;
B = 15 hours 5,300 cpm recovered.

C and D Oocyte oligo (dT) bound RNA labelled with ^{3}H-adenosine

C = 15 hours 1,850 cpm recovered;
D = 25 hours 2,600 cpm recovered.

The low incorporation of radioactive nucleoside into oligo (dT) bound RNA presented problems in the analysis of this RNA. In some experiments too few counts were incorporated to run sucrose gradients. The reason for the use of adenosine for these incorporation studies was to give a higher percentage incorporation into oligo (dT) bound RNA and also to label the poly(A). However, using radioactive adenosine could cause a complication of the kinetic picture if the poly(A) tail of pre-existing mRNA molecules turned over to any extent and an experiment was performed to test this. ^{3}H-adenosine labelled oligo (dT) bound RNA was digested with T_1 RNAse and the nuclease resistant poly(A) tracts were isolated on oligo (dT)-cellulose. The results of this experiment are given in Figure 3-7. Despite the large scatter, the percentage of the pre-digest incorporation which is T_1 RNAse resistant and which subsequently binds to oligo (dT)-cellulose is roughly constant over the 30 hour time period studied. This is true for both follicle sheath and oocyte oligo (dT) bound RNA.

Since the average size of the oligo (dT) bound RNA molecules is about 18S which corresponds to about 2,000 bases, and since there are approximately 100 A residues at the 3' end of these molecules (Rosbash & Ford, 1974), then the theoretical percentage adenosine in the poly(A) tail (assuming equimolar G, C, U and A throughout the rest of the molecule) is about 15% of the total adenosine which is close to the measured value. This shows that in these incorporation studies the oligo (dT) bound RNA molecules are being labelled throughout their entire length and not just in their poly(A) tails. This is emphasised by the fact that radioactive guanosine also labels oligo (dT) bound RNA (Chapter 4). A further conclusion is that there is no detectable shortening of the poly(A) tail of the newly synthesized oligo(dT) bound RNA molecules within the 30 hours of the experiment.

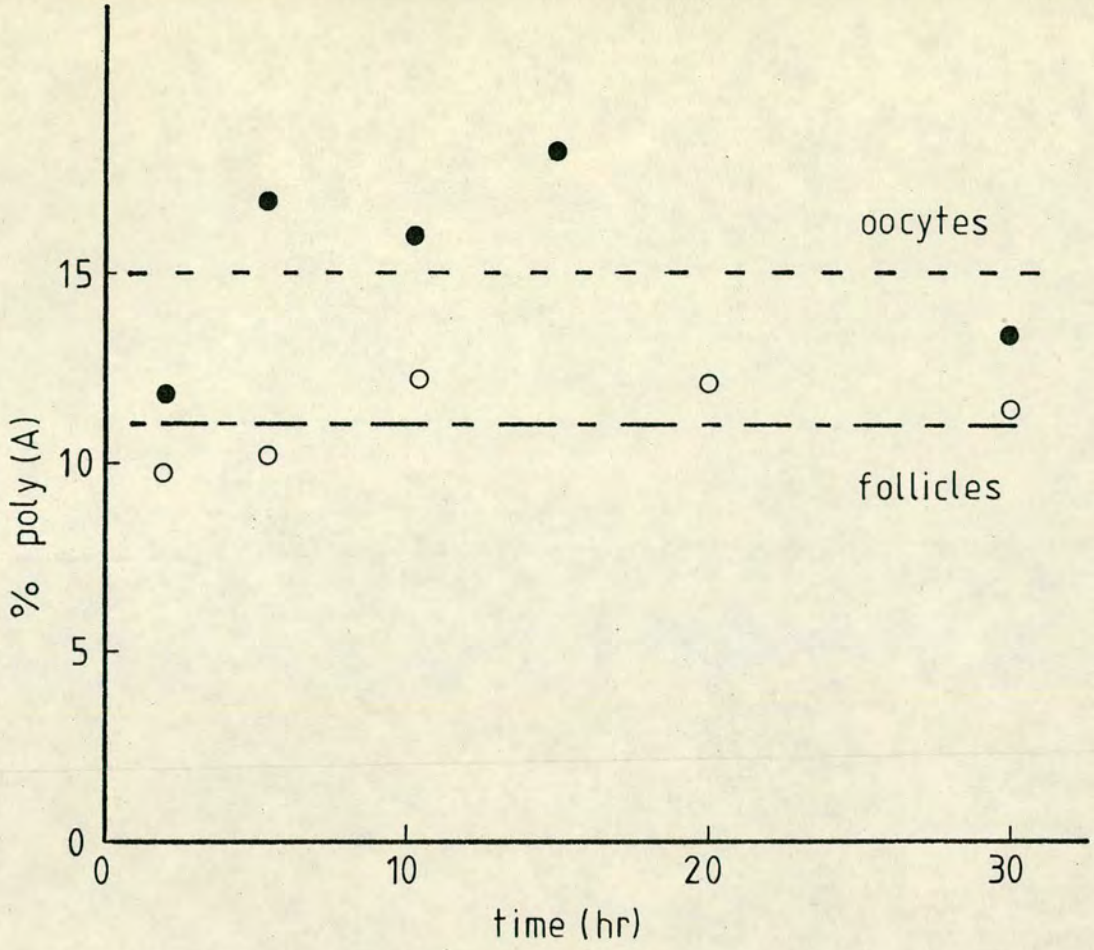


Figure 3-7 Incorporation into the poly(A) of oligo (dT) bound RNA from stage 6 oocytes and follicle sheaths of Xenopus laevis

Samples of oligo (dT) bound RNA prepared as described in Figure 3-5 were digested with RNAse T_1 and the poly(A) tracts were isolated as described in section (j) of materials and methods. For each time point the whole sample of poly(A) was precipitated with TCA and the radioactivity determined as detailed in materials and methods section (m). Recovery of total cpm after digestion was over 95% and 75-250 cpm were recovered in the poly(A) fractions from oocytes and 200-1,000 cpm were recovered in the follicle sheath poly(A) fractions. The background radioactivity was 11 cpm. The cpm recovered in the poly(A) is expressed as a percentage of the total cpm in the oligo (dT) bound RNA prior to digestion.

(●) oocyte poly(A), (○) follicle sheath poly(A)

(d) Preliminary germinal vesicle isolation results

Initial attempts to study the cellular distribution of the newly synthesized RNA involved manual isolation of the germinal vesicle by the method of Gall (1966) (materials and methods section (f)). At first the nuclei and cytoplasms were collected directly onto separate GF/C filters and washed with TCA in order to determine incorporation into RNA. As this method did not prove satisfactory in later attempts the usual Kirby's extraction procedure (materials and methods section (g)) was followed on the two cell fractions adding carrier RNA to the nuclear samples.

Uptake and incorporation as measured directly on GF/C filters

Figures 3-8 and 3-9 give the time courses of uptake of radioactive adenosine into stage 6 oocytes and their follicle sheaths as determined by counting the samples collected directly on GF/C filters. It is clear that the curves are in agreement with those given above. Figure 3-10 gives the time course of incorporation into TCA precipitable material by stage 6 oocyte germinal vesicles and cytoplasms collected on GF/C filters. The data shows wide scatter and even after 5 washes with 5% TCA the level of cpm incorporated is nearly an order of magnitude higher for the sum (cytoplasm and germinal vesicle) than previous experiments where RNA was extracted from intact oocytes. Bearing this in mind, the data suggests that incorporation into the germinal vesicle saturates whilst that in the cytoplasm increases approximately linearly.

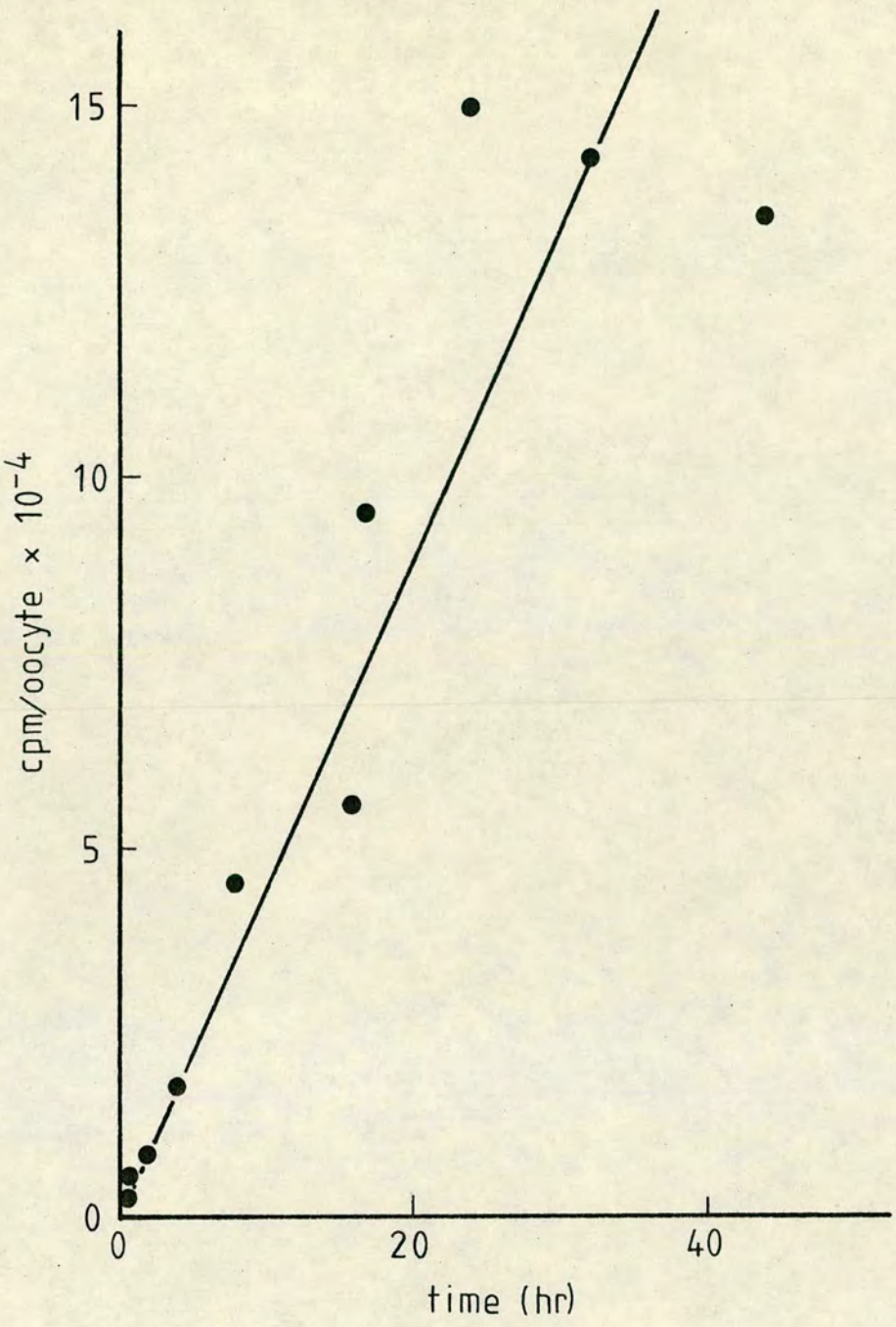


Figure 3-8 In vitro uptake of ^{3}H-adenosine by stage 6 oocytes measured directly on GF/C filters

Batches of 30 stage 6 oocytes manually stripped from an ovary, were incubated in 60 µl of MBX containing 1 mCi/ml 2-^{3}H-adenosine. At various times the incubation was stopped by washing the oocytes several times in MBX and the follicle sheaths were removed as described in section (e) of materials and methods. The oocytes were then placed directly onto GF/C filters at up to 5 oocytes per filter. To measure uptake the filters were dried and the radioactivity determined as detailed in materials and methods section (m). The data is expressed as total cpm per oocyte and is not corrected for quench, since no reliable value was obtained.

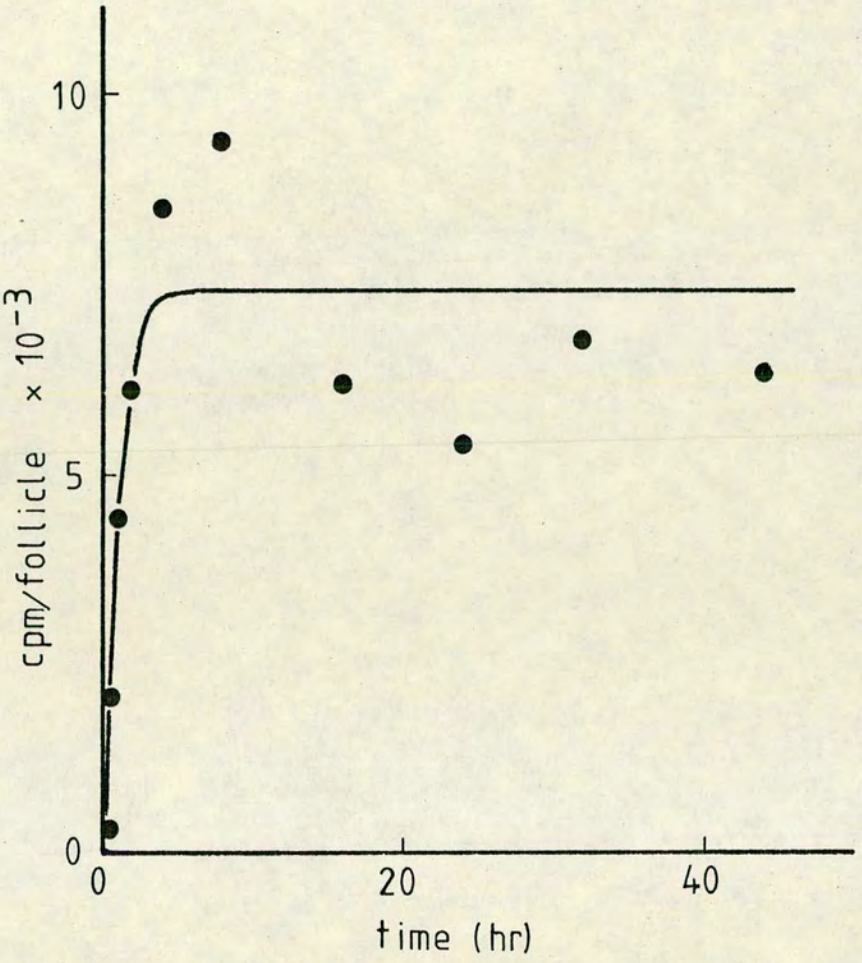


Figure 3-9 In vitro uptake of ^{3}H-adenosine by follicle sheaths measured directly on GF/C filters

The follicle sheaths from the oocytes in the experiment described in Figure 3-8 were collected directly onto GF/C filters (up to 5 follicle sheaths per filter) and the filters dried, the radioactivity determined and the data expressed exactly as in Figure 3-8.

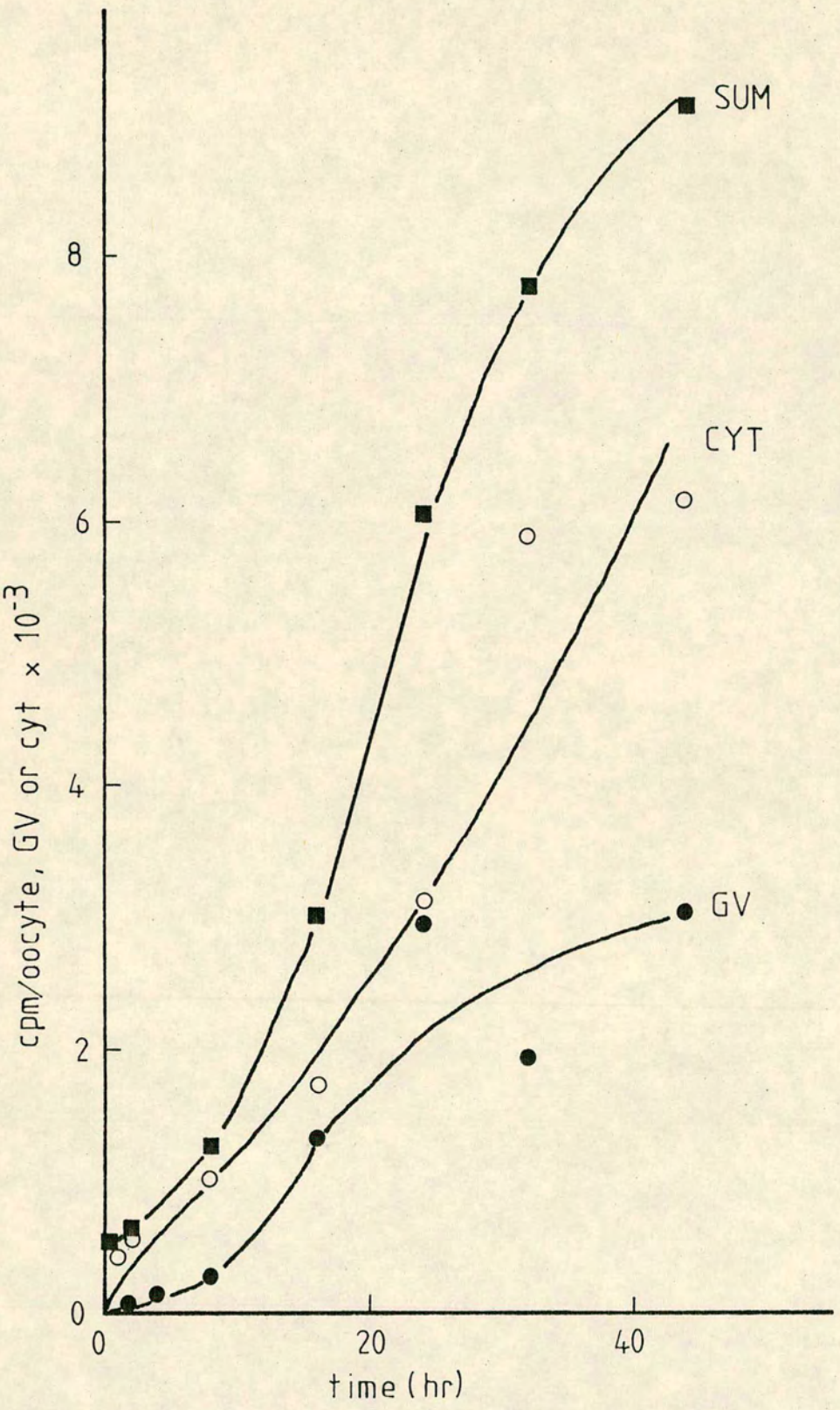

SUM
CYT
GV
8
6
4
2
0
0
20
40
time (hr)
cpm/oocyte, GV or cyt × 10-3

Figure 3-10 In vitro incorporation of ^{3}H-adenosine into TCA precipitable material by germinal vesicles and cytoplasms measured directly on GF/C filters

Germinal vesicles and cytoplasms were prepared from some of the oocytes of the experiment in Figure 3-8, as described in materials and methods section (f). Up to 5 germinal vesicles or cytoplasms were collected directly onto separate GF/C filters, dried, the radioactivity determined and the data expressed exactly as in Figure 3-8.

(●) Incorporation into the germinal vesicle.

(○) Incorporation into the cytoplasm.

(■) Sum of germinal vesicle and cytoplasm curves.

Figure 3-11A shows the uptake of radioactive guanosine by intact stage 6 oocytes and by isolated cytoplasms and Figure 3-11B gives similar results for the follicle sheaths and the germinal vesicles. The following conclusions can be drawn. Uptake of guanosine into the cytoplasm is linear and is approximately equal to that of the intact control oocytes. Uptake of guanosine into the germinal vesicles is also linear which suggests that the nuclear NTP precursor specific activity may be increasing linearly.

The rate of uptake by the germinal vesicle is about 1% of the total oocyte rate yet the volume of the germinal vesicle is about 7% of the oocyte volume in stage 6 oocytes (Scheer, 1973). This observation could be explained if some radioactivity leaked from the germinal vesicles during isolation. Figure 3-12 gives the time course of guanosine incorporation into total RNA for germinal vesicles, cytoplasms, control oocytes and follicle sheaths. The control oocyte curve is of the expected parabolic shape and the sum of the germinal vesicle and cytoplasm curves is in close agreement with it especially at later times where the count level is higher. Incorporation of guanosine into the cytoplasm is essentially similar to that for the total oocyte though at about $\frac{2}{3}$ the rate. Incorporation of guanosine into the germinal vesicles seems to level off at about 15-20 hours, the slow rise after 15 hours possibly reflects an increase in specific activity of this unstable class of RNA.

An attempt was made to prepare oligo (dT) bound RNA from the various oocyte fractions and the results are given in Figure 3-13. The incorporation of radioactive guanosine into oligo (dT) bound RNA in intact oocytes shows a similar S-shaped curve to that given earlier (Figure 3-6), however virtually no incorporation into

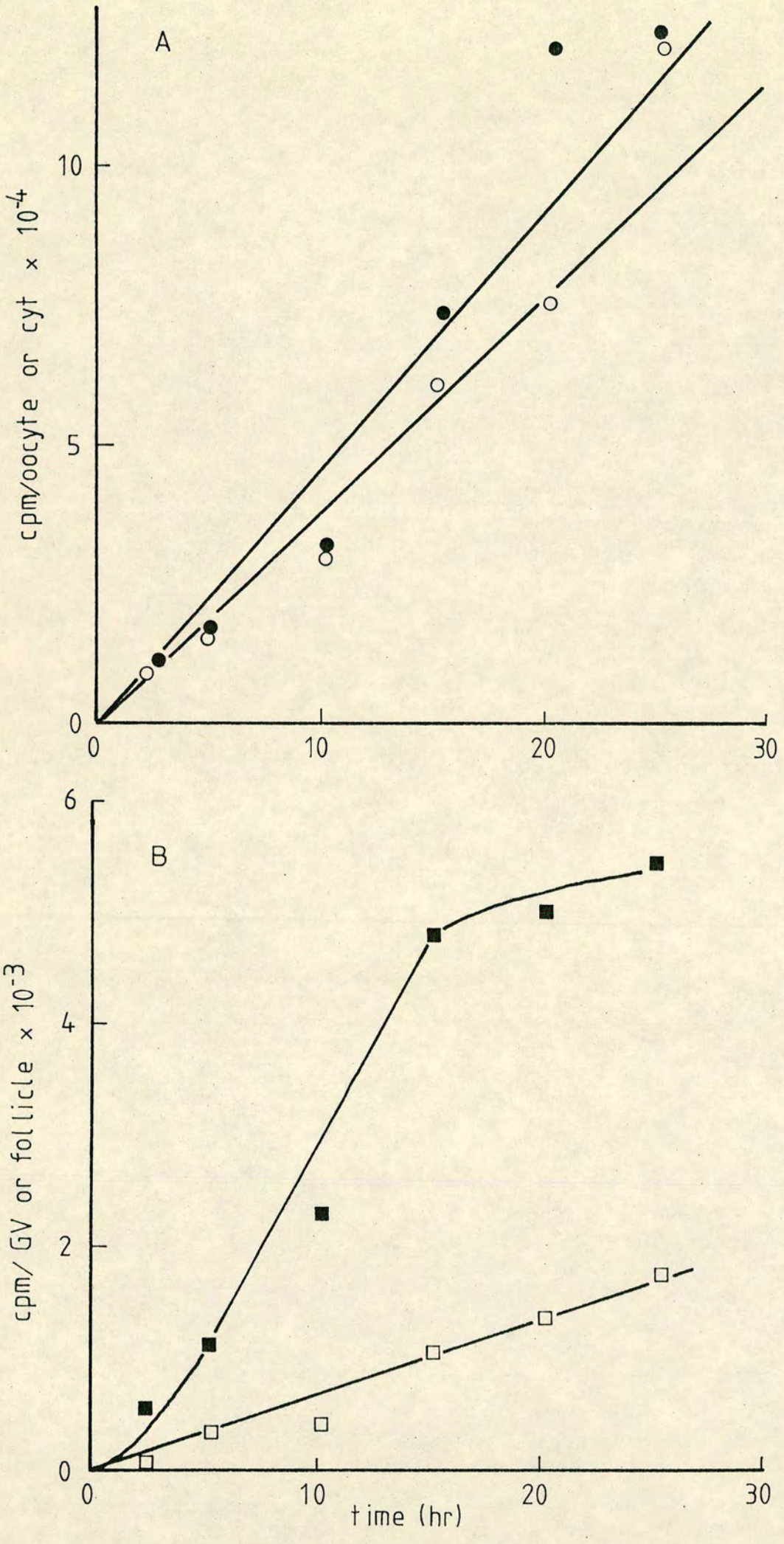

A
cpm/oocyte or cyt × 10⁻⁴
0
5
10
0
10
20
30
B
cpm/ GV or follicle × 10⁻³
0
2
4
6
0
10
20
30
time (hr)

Figure 3-11 In vitro uptake of ^{3}H-guanosine by oocytes, follicle sheaths, cytoplasms and germinal vesicles as measured by Kirby's extraction

Batches of 30 stage 6 oocytes, manually stripped from an ovary, were incubated in 60 µl of MBX containing 1 mCi/ml 8-^{3}H-guanosine. At various times the incubation was stopped by washing the oocytes several times in MBX and then the follicle sheaths were removed as described in materials and methods section (e). The follicle sheaths and some of the oocytes were collected separately in 4 ml of modified Kirby buffer, and homogenized. Germinal vesicles and cytoplasms were prepared from the remainder of the oocytes as described in materials and methods section (f) and these were also collected separately in 4 ml of modified Kirby buffer and homogenized. 100 µg of total ovary carrier RNA was added to the follicle sheath and the germinal vesicle samples. Aliquots (50 µl) of homogenate were placed directly onto GF/C filters, dried and the radioactivity determined as described in section (m) of materials and methods. This determination represents uptake of nucleoside from the medium and a correction was made for the quenching effect of Kirby buffer. RNA was extracted from the remaining homogenate (section (g) of materials and methods).

A (●) Uptake of ^{3}H-guanosine by control oocytes.
(○) Uptake of ^{3}H- guanosine by cytoplasms.

B (■) Uptake of ^{3}H-guanosine by follicle sheaths.
(□) Uptake of ^{3}H-guanosine by germinal vesicles.

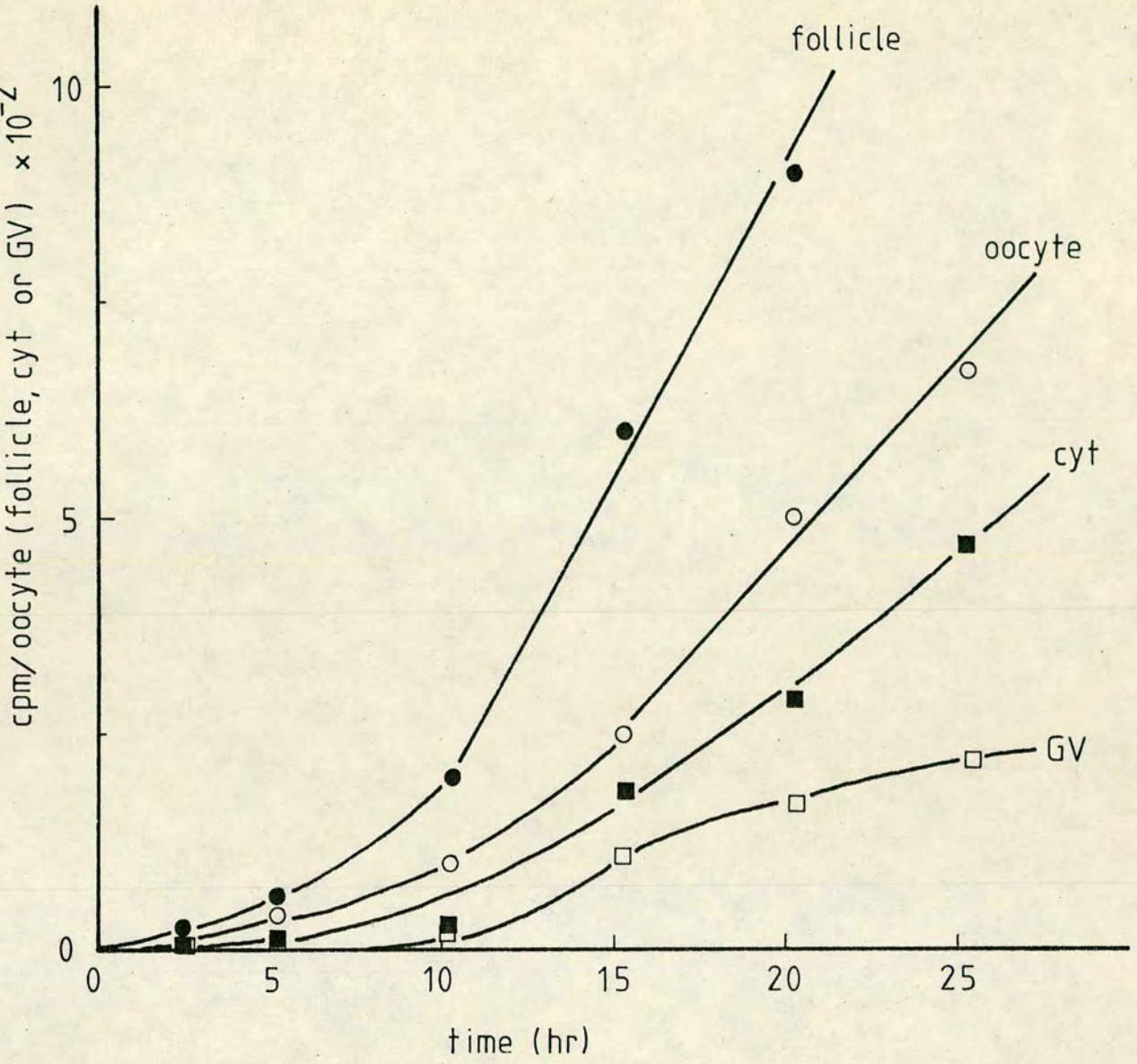


Figure 3-12 In vitro incorporation of ^{3}H-guanosine into total RNA by germinal vesicles, cytoplasms, control oocytes and follicle sheaths

Samples of total RNA prepared in the experiment described in Figure 3-11 were precipitated with TCA and the radioactivity determined as described in materials and methods section (m). The data is expressed as TCA precipitable cpm incorporated per follicle sheath, oocyte, cytoplasm or germinal vesicle.

(●) Incorporation of ^{3}H-guanosine into follicle sheath total RNA

(○) Incorporation of ^{3}H-guanosine into stage 6 oocyte total RNA

(■) Incorporation of ^{3}H-guanosine into cytoplasm total RNA

(□) Incorporation of ^{3}H-guanosine into germinal vesicle total RNA

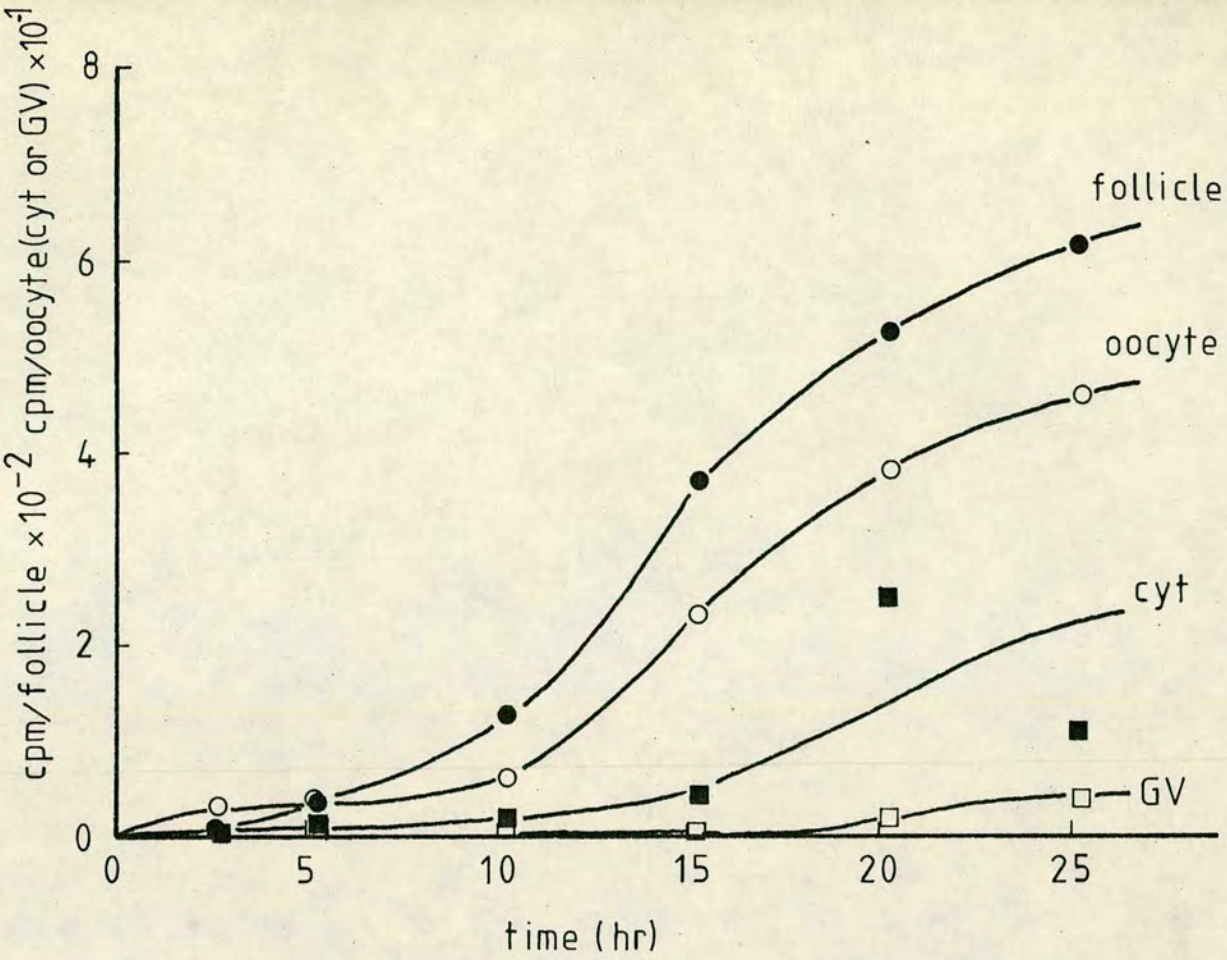


Figure 3-13 In vitro incorporation of ^{3}H-guanosine into oligo (dT) bound RNA by germinal vesicles, cytoplasms, control oocytes and follicle sheaths

Oligo (dT) bound RNA was prepared from the samples of total RNA which were labelled with ^{3}H-guanosine in the experiment described in Figure 3-11, as described in materials and methods section (h). Samples of oligo (dT) bound RNA were precipitated with TCA and the radioactivity determined as described in section (m) of materials and methods. Recovery of loaded cpm after oligo (dT)-cellulose chromatography was over 97%. The data is expressed as TCA precipitable cpm per follicle sheath, oocyte, cytoplasm or germinal vesicle.

(●) Incorporation of ^{3}H-guanosine into follicle sheath total RNA

(○) Incorporation of ^{3}H-guanosine into stage 6 oocyte total RNA

(■) Incorporation of ^{3}H-guanosine into cytoplasm total RNA

(□) Incorporation of ^{3}H-guanosine into germinal vesicle total RNA

germinal vesicle oligo (dT) bound RNA was observed during the 25 hours of the experiment. Incorporation into cytoplasmic oligo (dT) bound RNA was low and the time points looked unreliable. The sum of the germinal vesicle and cytoplasm curves did not generate the curve for the intact oocyte. From this experiment it was concluded that during the germinal vesicle isolation procedure RNA, and particularly oligo (dT) bound RNA, was either lost to the isolation medium or was degraded.

It was obvious that before any reliable data could be obtained on the kinetics of synthesis of oligo (dT)bound RNA by the intact oocyte, let along by oocyte fractions, it would be necessary to try and improve both the oligo (dT)-cellulose procedure and the method of germinal vesicle isolation.

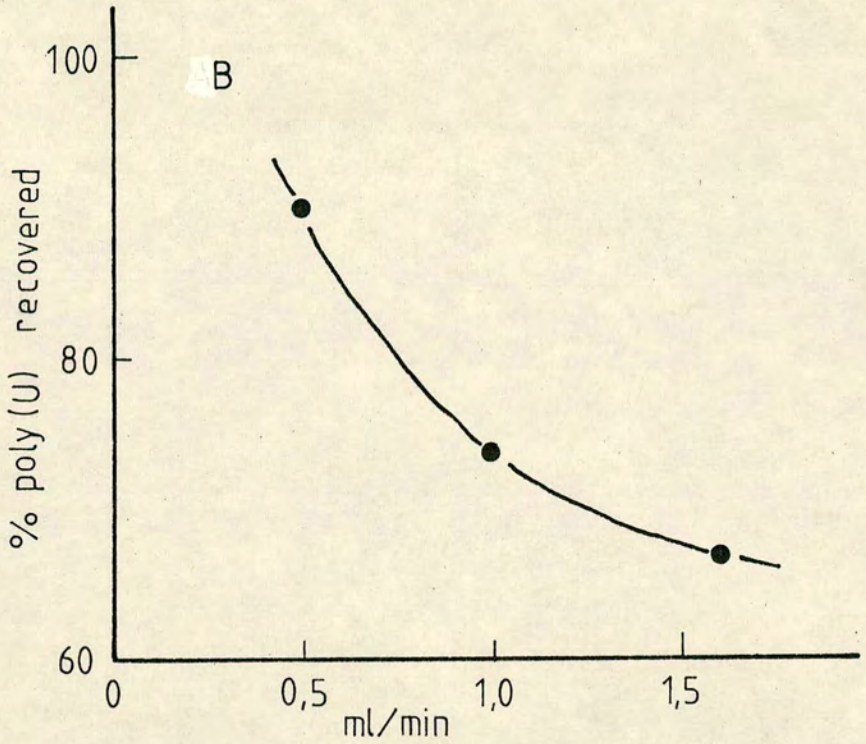
(e) Improvement of methods

Oligo (dT)-cellulose procedure

It was found that using the initial oligo (dT)-cellulose procedure (materials and methods section (h)) the degree of contamination of the oligo (dT) bound RNA by rRNA was excessive and variable and therefore the following variations to the initial method were tried.

Number of reloadings

The percentage of the total poly(U) binding activity of Xenopus laevis total ovary RNA recovered in the oligo (dT) bound fraction as a function of the number of times loaded onto the column at constant flow rate is given in Figure 3-14A. It is clear that very little increase in the percentage of the total poly(U) binding activity recovered in the bound occurs after 3 cycles of loading.


B
100
80
60
% poly(U) recovered
0
0,5
1,0
1,5
ml/min

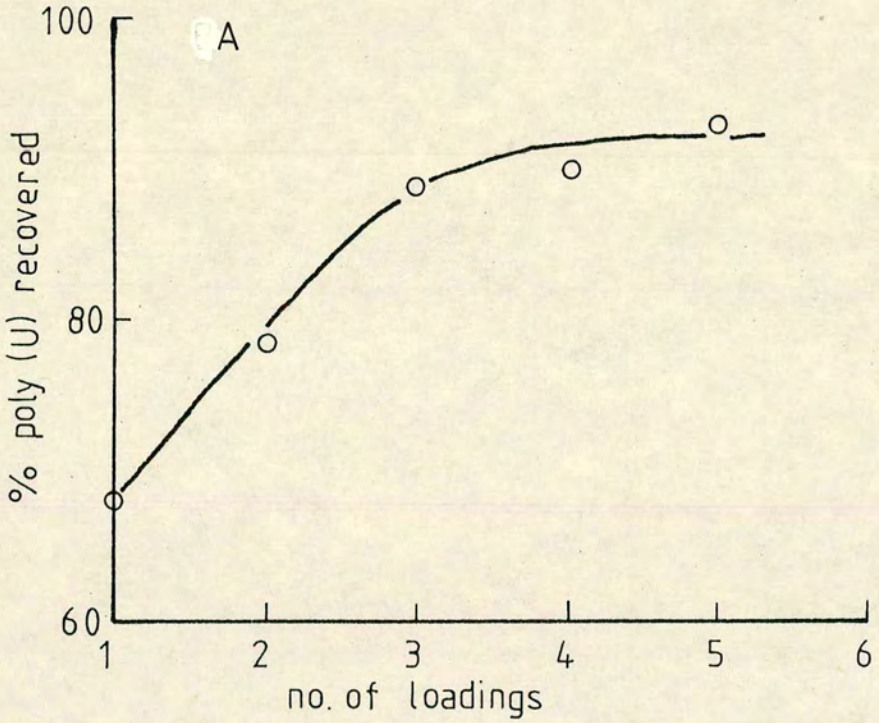

A
100
80
60
% poly (U) recovered
1
2
3
4
5
6
no. of loadings

Figure 3-14 Effect of number of reloadings and flow rate on the efficiency of oligo (dT)-cellulose affinity chromatography

Using the initial method outlined in materials and methods section (h) samples of total ovary RNA were loaded onto oligo (dT)-cellulose columns to obtain a bound fraction. The poly(U) binding activity of the initial sample, the final void fraction and the final bound fraction were determined as described in section (k) of materials and methods. The results are expressed as the percentage of the total poly(U) binding activity recovered which is present in the bound fraction. Over 98% of the loaded poly(U) binding activity was recovered from the column.

(A) Samples were reloaded onto the column 1-5 times, washing the column with an equal volume of binding buffer between each reloading step. The flow rate was constant at 1.0 ml/min.

(B) Using 3 loadings, samples were passed through the column at different flow rates between 0.5 and 1.5 ml/min.

Flow rate

Using 3 loadings, Figure 3-14B gives the percentage of the total poly(U) binding activity recovered in the bound fraction using different flow rates. As the flow rate decreases the percentage of the poly(U) binding in the bound increases. There are obviously practical limits to the minimum flow rate possible.

Non-specific binding

Figure 3-15 presents the results of experiments to see what effect flow rate and the number of reloadings has on the sucrose gradient profiles of the oligo (dT) bound fraction of labelled total ovary RNA. A comparison of Figures 3-15B and 3-15C with 3-15A shows that although the recovery of the heterogeneous oligo (dT) bound RNA increases a little by reloading or using a slow flow rate the amount of 18S and 28S rRNA contamination also increases. Figure 3-15D shows the results of rebinding this contaminated RNA to oligo (dT)-cellulose to produce an oligo (dT) bound-bound fraction. The 18S and 28S contamination is reduced by the second binding step. A slight reduction in the amount of heterogeneous RNA that binds may also result.

The reaction of poly(A) with oligo (dT) is a hybridization reaction which might therefore be expected to go further to completion if the time of reaction is increased. Both slowing the flow rate of the column and increasing the number of loadings should in effect increase the time of reaction and produce a more efficient binding of poly(A)$^+$RNA. This is observed, but as can be seen from the gradients only at the expense of the purity of the preparation, non-specific binding is also favoured by these procedures. If, however, the first preparation of oligo (dT) bound RNA (which is greatly enriched

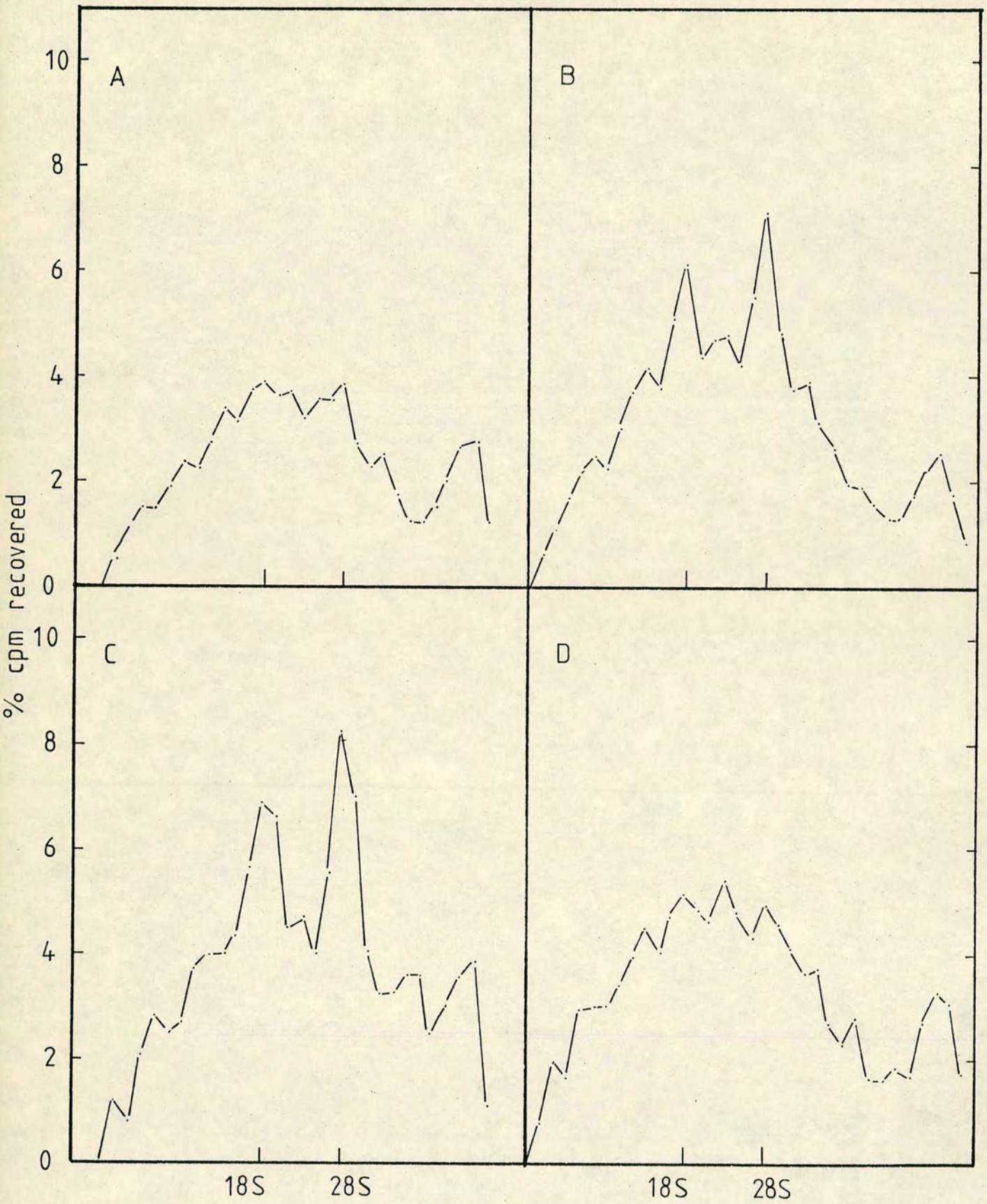

A
B
C
D
% cpm recovered
0
2
4
6
8
10
18S
28S
18S
28S

Figure 3-15 Effects of modifications to the oligo (dT)-cellulose procedure on the purity of the bound RNA

Samples of oligo (dT) bound RNA were parpared from total ovary RNA using the conditions described in Figure 3-14 and/or materials and methods section (h). The samples were sedimented in 11.5 ml linear 7-30% sucrose gradients in NETS buffer at 40,000 rpm for $4\frac{1}{2}$ hours at 25°C in an MSE 6 x 14 ml Ti rotor. The gradients were analysed as described in materials and methods section (i) and aliquots of each gradient fraction were hybridized to ^{3}H-poly(U) as described in section (k) of materials and methods. The results are expressed as the percentage of the total ^{3}H-poly(U) binding recovered which is present in each fraction. Approximately 10,000 cpm of poly(U) binding activity were loaded and recovered from each gradient.

(A) Oligo (dT) bound RNA prepared by the initial method (materials and methods, section (h)).

(B) Bound RNA prepared using 5 reloadings (Figure 3-14A)

(C) Bound RNA prepared using 0.5 ml/min flow rate (Figure 3-14B)

(D) Bound RNA prepared using the standard method (materials and methods, section (h)).

for poly(A)$^+$RNA) is subjected to a further round of hybridization the non-specific binding can be reduced (Figure 3-15D). If the non-specific binding of rRNA is due in any part to its interaction with poly(A)$^+$RNA then it would be reasonable to expect that denaturing the RNA prior to its loading onto the column would help to reduce this non-specific binding. Further, if unlabelled carrier RNA can be added to the first oligo (dT) bound RNA before it is denatured and reloaded then any reassociation of the labelled poly(A)$^+$RNA with rRNA which might occur will be competed out by the unlabelled carrier RNA added.

In conclusion, it was decided to adopt the following standard oligo (dT)-cellulose procedure in future experiments (materials and methods section (h)), the important points being:

(1) Heat the sample to 65° for 2 minutes and chill.

(2) Load the sample in a small volume and reload two further times, washing the column with binding buffer between loadings.

(3) Make the bound 0.4 M NaCl, add cold carrier RNA if possible, heat to 65° for 2 minutes and chill.

(4) Bind this fraction once to a regenerated oligo (dT)-cellulose column.

Since this standard method has been used throughout the rest of the investigation the oligo (dT) bound-bound RNA produced is simply referred to as oligo (dT) bound RNA.

Germinal vesicle isolation procedure

In the preliminary experiments involving germinal vesicle isolation outlined above, concern was shown that some RNA isolated from the germinal vesicle might be degraded or lost to the medium

during the isolation procedure. Attempts were therefore made to improve the basic isolation procedure which was essentially the method of Gall (1966). It was reasoned that degradation of the RNA might be prevented by including nuclease inhibitors in the isolation medium. The problem of leakage from the germinal vesicles was not easy to overcome. Obviously the length of time that the isolated germinal vesicle spends in the medium is important and should be kept to an absolute minimum. Secondly, germinal vesicle isolation removes the nucleus from a high molecular weight environment and places it in a simple salt medium. Leakage under these conditions might be expected to be very rapid. If the molecular environment of the isolated germinal vesicle could be made in some way to resemble that in the intact oocyte leakage might be reduced. A new medium was made up containing 2% polyvinyl pyrrolidone, 0.5 mM DTT, 50 μg/ml dextran sulphate, 50 μg/ml spermine, 100 mM KCl, 20 mM NaCl, 0.8 mM $CaCl_2$, 2 mM $MgCl_2$, buffered to pH 7.0 with HEPES.

Figure 3-16 shows an absorbance scan of an SDS/acrylamide gel of total RNA extracted from germinal vesicles isolated in this new medium, and also a similar gel of total cytoplasmic RNA for comparison. The profile for cytoplasmic RNA shows distinct peaks of 28S and 18S rRNA and no obvious degradation. The germinal vesicle RNA has a component of slow running high molecular weight heterogeneous RNA above which are seen the peaks of the 32S and 20S rRNA precursors, which run slower than the 28S and 18S rRNA of the cytoplasm, indicating that they are not degraded. Some 18S RNA is present in the germinal vesicle gel as a shoulder on the peak of 20S RNA. By area, this 18S RNA does not exceed 25% of the amount of 18S RNA in the cytoplasm sample. Therefore assuming that all the 18S RNA in the germinal vesicle

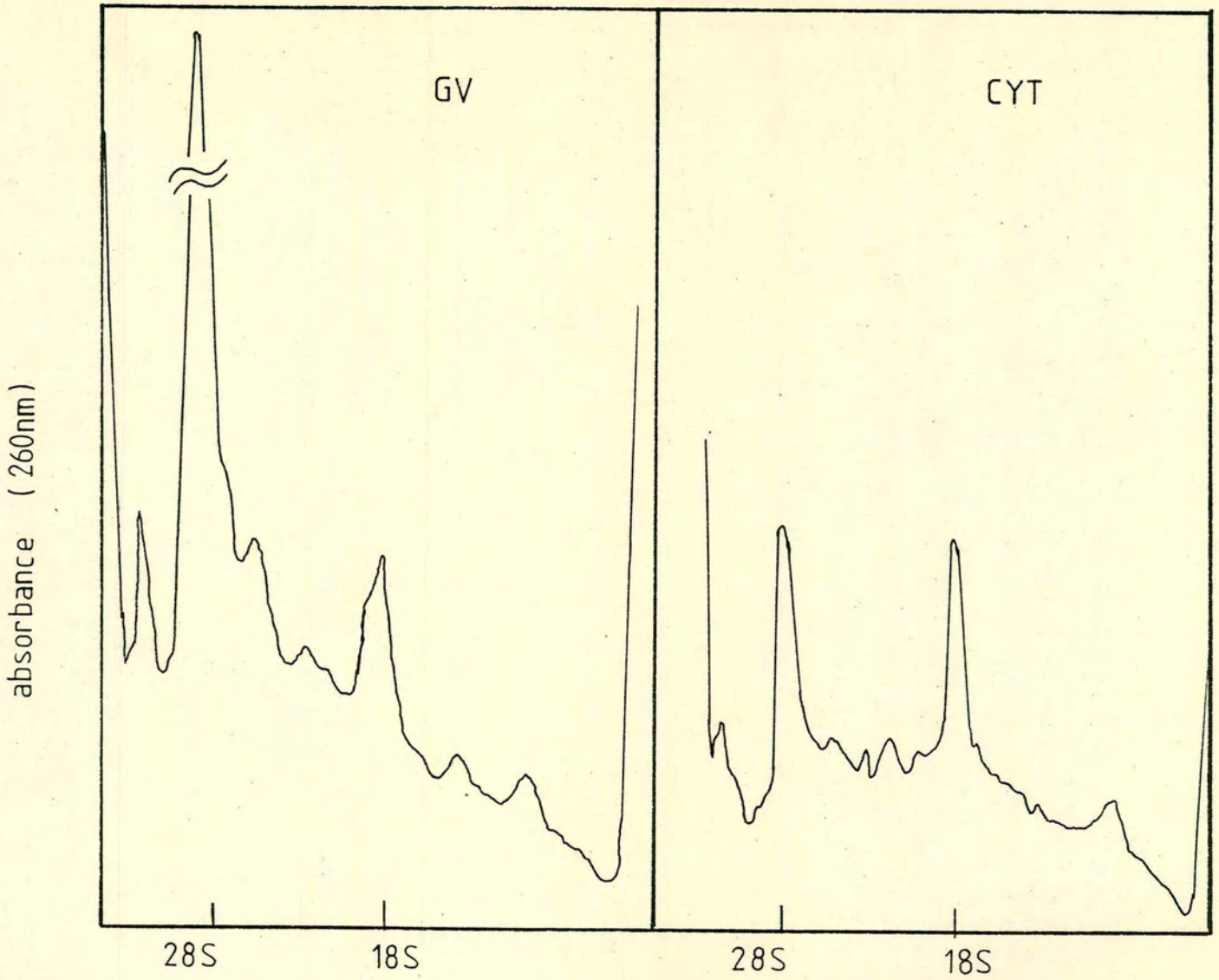

GV
CYT
absorbance (260nm)
28S
18S
28S
18S

Figure 3-16 Absorbance scan of total RNA from germinal vesicles and cytoplasms

500 germinal vesicles and 60 cytoplasms were prepared as described in materials and methods section (f) and were collected in 4 ml of modified Kirby buffer. Total RNA was extracted as described in section (g) of materials and methods and the optical density measured. To calculate the amount of RNA present 1 OD unit was taken to be equivalent to 40 μg RNA. Samples of total RNA were analysed by electrophoresis on 2.3% SDS/acrylamide gels and the absorbance at 260 nm measured as described in materials and methods section (l).

Sample	Total number present	Optical density (OD)	RNA content ng/cyt or GV	OD loaded on gel	Number of cyt or GV loaded	Ratio of cyt:GV loaded
Cytoplasm	60	5.78	3,850	0.355	3.7	1
Germinal vesicle	500	0.62	50	0.400	323	87

sample is due to contamination and also assuming that twice this amount of 28S RNA will further contaminate the germinal vesicle preparation, then from the known number of germinal vesicles and cytoplasms one can calculate the amount of 18S RNA in the germinal vesicle. There is 25% as much 18S RNA in 87 germinal vesicles as in 1 cytoplasm (Figure 3-16). The amount of 18S RNA in a stage 6 oocyte is 1250 ng (Rosbash & Ford, 1974). 1250 x 25% ÷ 87 = 3.6 ng of 18S RNA. Therefore approximately 10 ng of 18S and 28S RNA contaminates the germinal vesicle RNA. The measured amount of RNA per germinal vesicle in this experiment was 50 ng and consequently the germinal vesicle preparation is estimated to be 80% pure. This calculation, taken together with other measurements of about 50 ng for the RNA content of a stage 6 Xenopus laevis germinal vesicle (Burzio & Koide, personal communication), and with the observation presented later, that in labelling experiments over 40% of the radioactivity present in germinal vesicle total RNA after 43 hours is in heterogeneous RNA (cf. 20% for cytoplasm) strongly suggests that the germinal vesicle RNA isolated in this modified medium is not degraded and represents a substantial proportion of the total nuclear RNA.

Labelling of oocytes

A major stumbling block in discussing the kinetics of synthesis of the various classes of RNA made by Xenopus laevis oocytes was the fact that the specific activity of the radioactive precursor was not constant. At best it was assumed to be increasing linearly, since the uptake of radioactive nucleoside from the medium was linear. There are a number of problems encountered in trying to obtain kinetic data under these conditions. Firstly, at early times little

nucleoside has penetrated the oocyte and the RNA precursor specific activity is so small that incorporation is almost undetectable. This would mean that classes of RNA which turned over rapidly would not be detected. Secondly, although in theory it might be possible to describe fairly accurately a situation where only one class of RNA was being synthesised, be it completely stable or completely unstable, the fact that the specific activity of the precursor is rising at an unknown rate makes the expected degree of deviation of the time course from linearity or saturation unpredictable. Thirdly, scatter in the data points which is quite large in these kinds of experiments, may permit one to draw almost any curve. This lack of precision cannot completely be overcome by increasing the number of time points since there are a number of practical factors which impose a relatively low limit on the possible number of data points.

One obvious solution to these problems would be to apply the technique of microinjection. Theoretically, microinjection would have the following advantages, providing the injected oocyte did not leak cellular contents and maintained its pre-injection synthetic activity. Almost immediately after injection a much higher specific activity than could be achieved even with long incubation times would be established and this specific activity should remain constant for a length of time after injection which would depend on the precursor pool size and the rate of use of the precursor NTP. This constant specific activity would greatly facilitate interpretation of the kinetic curves and RNA species turning over rapidly may be detectable. If the immediate metabolic precursor of RNA, the NTP, is injected then a knowledge of its specific activity together with a knowledge of the oocyte NTP pool size would allow one to estimate the initial specific

activity of that NTP within the oocyte. This would be an important value to have as it would allow quantitation of the rates of synthesis of RNA.

(f) Discussion

In this Chapter preliminary attempts to study RNA synthesis in vitro in stage 6 Xenopus laevis oocytes were described. Some time was devoted to developing the conditions of incubation of the oocytes and it was concluded that where possible fresh oocytes should be incubated with their follicle sheaths intact and these must be dissected off manually at the end of the incubation. Adopting this procedure, time courses of nucleoside uptake and incorporation into oocytes and their follicle sheaths were obtained. It was shown that uptake of nucleoside from the medium was linear in oocytes but saturated in follicle sheaths. Incorporation of nucleoside into TCA precipitable material was demonstrated to occur actively throughout the time courses showing that oocytes and follicle sheaths manipulated in the above manner remained healthy. The incorporation was shown to be into RNA which was analysed on sucrose gradients and 4S/5S, 18S, 28S, 40S, and heterogeneous RNA species were all labelled.

Oligo (dT) bound RNA was also shown to be labelled during these experiments and this labelling was not simply due to turnover of the poly(A) tails of these molecules. Analysis of this oligo (dT) bound RNA was difficult due to its variable contamination with rRNA and low incorporation. This prompted efforts to improve the oligo (dT)-cellulose procedure which had some degree of success.

Initial attempts to study RNA synthesis in nuclear and cytoplasmic fractions of the oocyte were hampered by possible degradation and loss of the RNA in particular from the nuclear fraction. Although some

results were obtained it was found necessary to improve the nuclear isolation conditions. Evidence was presented to show that this was largely achieved.

Limitations of the initial labelling technique in terms of a kinetic analysis were discussed and it was resolved to apply the technique of oocyte microinjection to overcome these limitations. The results gained using this technique with particular emphasis on the synthesis of poly(A)$^+$RNA are the subject of the next Chapter.

CHAPTER 4

RNA Synthesis in Microinjected Stage 6 Oocytes

(a) Introduction

The information about RNA synthesis gained by incubating stage 6 Xenopus laevis oocytes in radioactive nucleosides is consistent with earlier work (LaMarca et al., 1973, 1975; Colman, 1974), and confirms that oocytes of this stage are active in the synthesis of rRNA, which appears to be relatively stable, 4S and 5S RNA and heterogeneous RNA. Also in Chapter 3 a class of newly synthesized RNA representing approximately 5% of the total incorporation was isolated by hybridization to oligo (dT)-cellulose and was shown to sediment in a heterogeneous manner on sucrose gradients. The sedimentation properties of this class of RNA were similar to those reported for the stored pool of poly(A)$^+$RNA from stage 6 oocytes of Xenopus laevis (Rosbash & Ford, 1974). About 15% of the radioactive adenosine incorporated into this RNA was stable to digestion with RNAse T_1 and could still hybridize to oligo (dT)-cellulose. These properties of this class of newly synthesised RNA are similar to those of eucaryotic mRNA in general (Greenberg, 1975).

The experiments of Chapter 3 were unable to describe accurately the kinetics of synthesis of this class of RNA and therefore knowledge of its stability is unknown. In addition the experiments to determine the cellular localization of the newly synthesized RNA did not give clear answers. By careful application of the techniques of oocyte microinjection and germinal vesicle isolation both of these problems have been clarified and the results are the subject of the first part of this Chapter.

The second part of Chapter 4 deals with experiments which try to establish whether or not poly(A)$^+$RNA synthesized in microinjected stage 6 oocytes is a product of transcription of the nuclear genome.

(b) Kinetics of RNA synthesis in intact stage 6 oocytes

Application of the microinjection technique

The microinjection technique detailed in materials and methods section (d) can be used to give an estimate of the rate of RNA synthesis in oocytes. Certain assumptions have been made in order to generate values for the rate of RNA synthesis in stage 6 oocytes and are given below. A known quantity of radioactive precursor (ATP or GTP) was dried down, redissolved and injected into the oocytes. It was assumed that the injected NTP mixed rapidly and completely with the cellular pool of that NTP. Knowing the quantity and specific activity of the injected NTP and its cellular pool size it was possible to estimate the initial specific activity of that NTP in the oocyte.

The quantity of NTP injected into the oocytes was estimated by summing the radioactivity recovered from the oocytes and their incubation medium at the end of the experiment and by using the manufacturers specific activity data. The values obtained show that when using low specific activity NTPs the amount injected was 30% or less of the pool size. Using high specific activity NTPs this value is 3% or less.

During the course of the longest experiments less than 15% of the pool would be incorporated into RNA using the rates of synthesis given by LaMarca et al. (1973). If the pool size is maintained the initial NTP specific activity may decrease by a similar amount due to this process.

Leakage of injected radioactivity in these experiments was variable but seldom exceeded 20% even after the longest incubation times. Again, if the pool is maintained this process could result in a further decrease in the NTP specific activity. In some

experiments the longest time points did appear to be too low and since this occured generally when leakage was high it must be concluded that the specific activity of the injected NTP may not remain constant throughout the whole duration of the longest experiments. Figure 4-1 shows that in oocytes which did not appear to leak the total soluble cpm/oocyte remains constant throughout the experiment. This supports the hypothesis that the NTP precursor specific activity remains constant throughout incubations of up to five days.

Woodland and Pestell (1972) have determined the pool sizes of NTPs in stage 6 oocytes from different Xenopus laevis females and LaMarca et al. (1973) have measured the pool size of GTP in similar oocytes. It is clear that considerable variation exists between females but the latter authors used their value of 256 ± 76 pmoles for the pool size of GTP in their RNA synthetic rate calculations. This value has been used here in estimating the rates of RNA synthesis after injection of radioactive GTP. In the case of injected ATP a pool size of 600 pmoles has been assumed (Woodland & Pestell, 1972).

It must be emphasised that without accurate knowledge of the pool size of the NTP in oocytes from any particular female comparison of the estimated rates of RNA synthesis between females is not a valid procedure, however, comparison of the estimated rates of synthesis of different classes of RNA within the same oocyte is more valid since it depends on the assumption that compartmentalization of the intracellular pool of NTP does not occur.

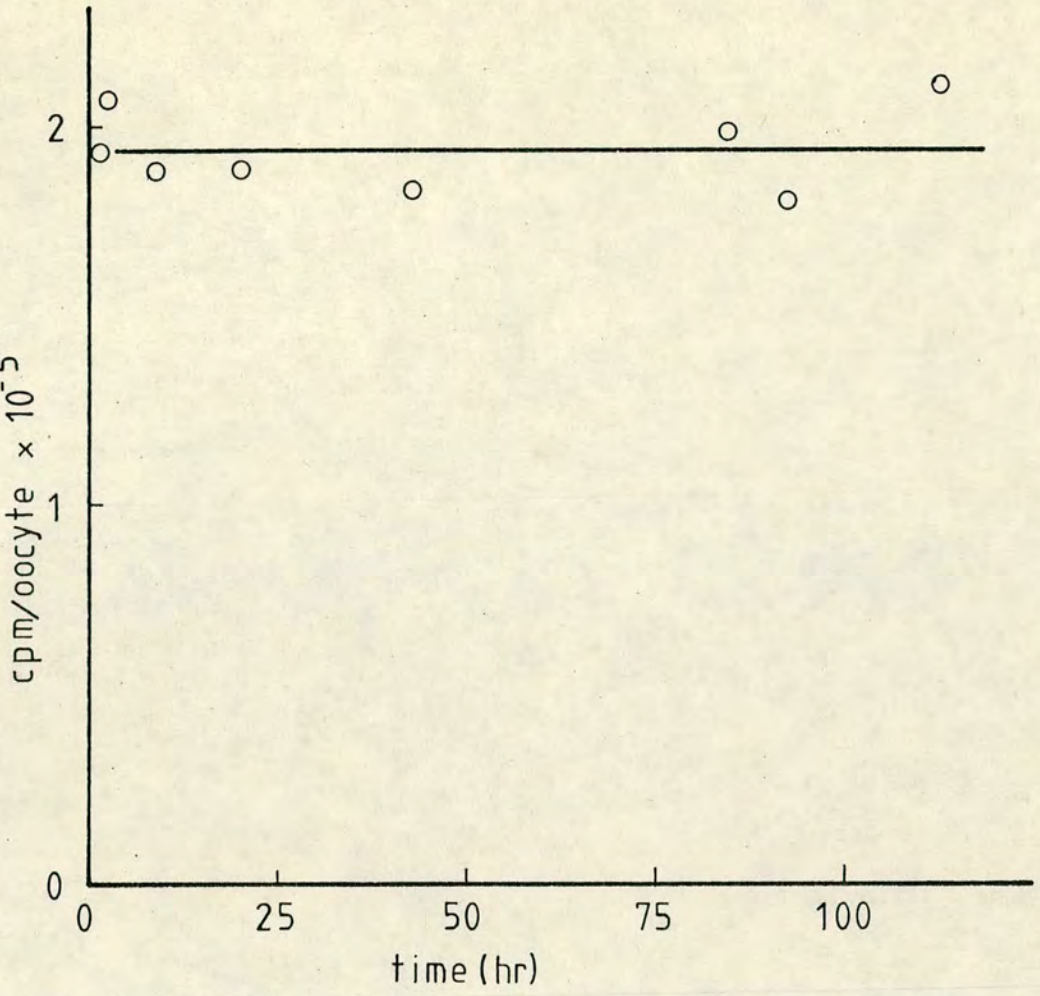


Figure 4-1 Total cpm in homogenates of microinjected stage 6 oocytes

Approximately 200 stage 6 oocytes were manually stripped from an ovary and microinjected in batches of 25, with 1 μCi of ^{3}H-GTP per oocyte as detailed in materials and methods section (d). The oocytes were incubated for various times in small petri dishes containing 2.5 ml MBX after which time the oocytes were washed several times, the follicle sheaths removed (materials and methods section (e)) and the oocytes collected and homogenized in 2 ml of modified Kirby buffer. Aliquots of homogenate were dried directly onto GF/C filters and the total radioactivity determined as described in materials and methods section (m), RNA being extracted from the remainder of the homogenate. The total radioactivity in the homogenate was corrected for the quenching effect of modified Kirby buffer and the results are expressed as cpm/oocyte.

Incorporation into total RNA

Figure 4-2 gives the kinetics of incorporation of various radioactive NTPs into total RNA in stage 6 oocytes from different Xenopus laevis females. The data supports the conclusions drawn in the previous Chapter showing that there is a linear incorporation of label into total RNA for at least 110 hours of incubation in vitro. A closer examination of these curves shows that in all cases where sufficient early time points have been taken a small positive intercept on the y-axis is produced if the line is extrapolated. This discontinuity in the incorporation curve is not due to the failure to deduct zero time incorporation and in fact the deduction of zero time incorporation may well have minimized the magnitude of this discontinuity. In correcting for zero time incorporation the TCA precipitable radioactivity in the zero time sample was subtracted from the TCA precipitable radioactivity in the other time points prior to their conversion to pmoles/oocyte incorporated. However, when samples of TCA precipitable material extracted at zero time were run on sucrose gradients the radioactivity invariably ran at the top of the gradient. This suggests that zero time values of incorporation are overestimates which may result from unphysiological association of newly injected NTP with TCA precipitable macromolecules.

In view of this, the incorporation curves for total RNA clearly demonstrate the existence of an unstable component in the stage 6 oocyte total RNA and this conclusion is in agreement with LaMarca et al., (1975). Table 4-1 presents values for the rates of RNA synthesis obtained in several experiments of this type. The stable rate of RNA synthesis is that corresponding to the linear part of the curve

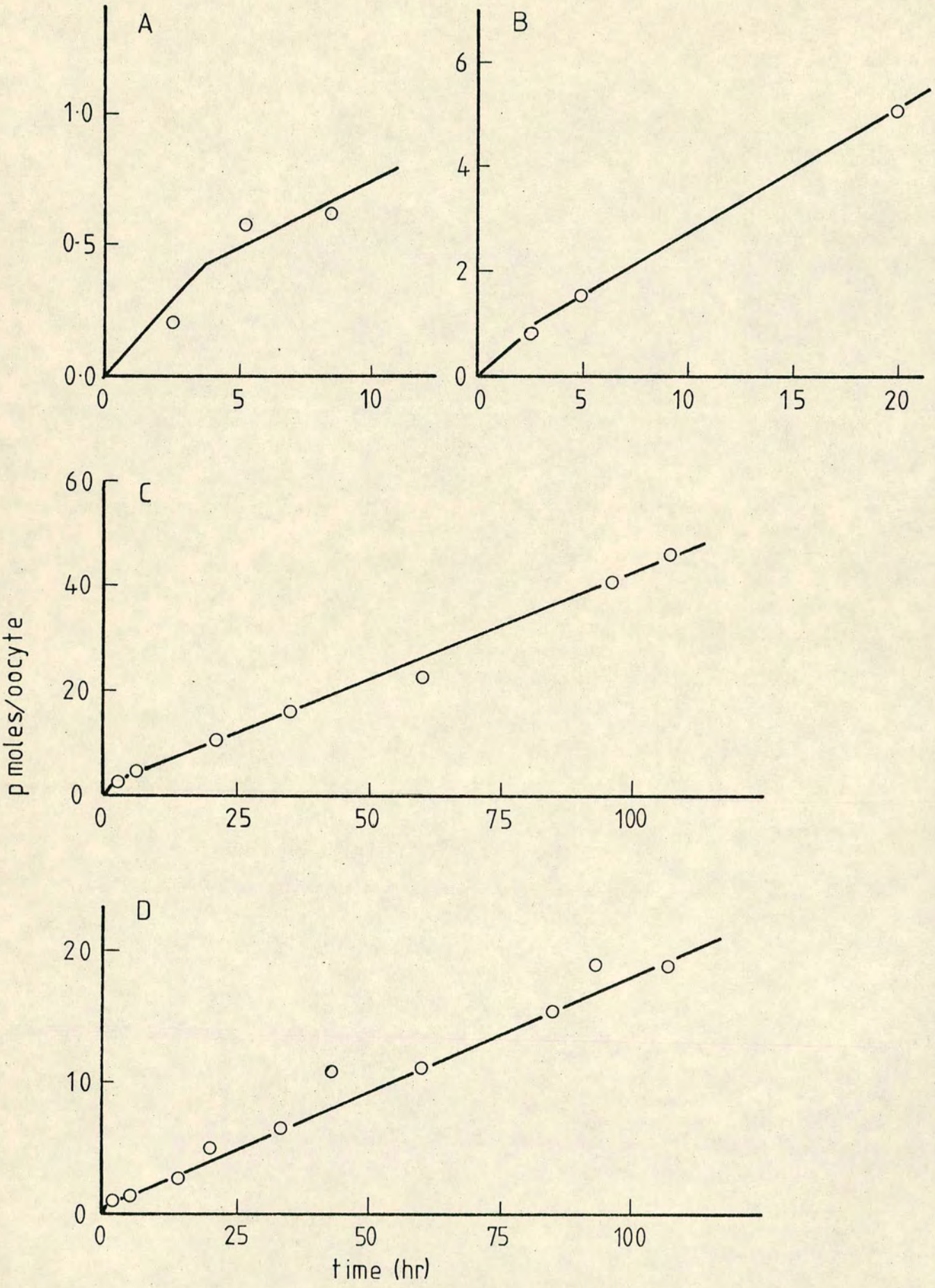

A
1·0
0·5
0·0
0
5
10
B
6
4
2
0
0
5
10
15
20
C
60
40
20
0
0
25
50
75
100
p moles/oocyte
D
20
10
0
0
25
50
75
100
time (hr)

Figure 4-2 Incorporation into total RNA in microinjected stage 6 oocytes

Several experiments of the type described in Figure 4-1 were performed using various radioactive NTPs. Samples of total RNA were precipitated with TCA and the radioactivity determined as described in materials and methods section (m). In all the experiments both the total radioactivity in the oocyte homogenates (Figure 4-1) and in the incubation dishes were determined and summed to give a value for the total cpm/oocyte injected. This was used to calculate an initial specific radioactivity (SA) of NTP in the oocyte by dividing by the pool size of the NTP injected (256 and 600 pmoles for GTP and ATP respectively) making an allowance for the quantity of NTP injected. The incorporation of NTP in cpm/oocyte was converted to pmoles of NTP/oocyte using the initial specific radioactivity estimated in this manner. The cpm incorporated at zero time has been subtracted from each time point.

(A) Incorporation of ^{3}H-ATP, SA about 1,000 cpm/pmole.

(B) Incorporation of ^{3}H-GTP, SA about 1,200 cpm/pmole.

(C) Incorporation of ^{32}P-GTP, SA about 5,000 cpm/pmole.

(D) Incorporation of ^{3}H-GTP, SA about 1,200 cpm/pmole.

In both C and D the lines have been fitted by linear regression analysis and the correlation coefficients (r) and equations generated are:

(C) $y = 0.4x + 1.3, \quad r = 0.996$

(D) $y = 0.2x + 0.3, \quad r = 0.995$

Table 4-1 Comparison of rates of incorporation of injected NTP into RNA

		A	B	C
Exp.	Label	Estimated Initial Rate pmoles/oocyte/hr	Stable Rate pmoles/oocyte/hr	Initial Rate / Stable Rate
1	^{3}H-GTP	0.23	0.11	2.11
2	^{3}H-GTP	0.19	0.09	2.24
3	^{3}H-GTP	1.25	0.43	2.94
4	^{32}P-GTP	0.80	0.40	2.00
5	^{32}P-ATP	0.50	0.23	2.17

Data compiled from several experiments of the type described in Figure 4-2. The stable rate (column B) corresponds to the linear part of the curve. The initial rate (column A) is obtained using the first time point only and therefore the initial rates are minimum estimates. Column C is the ratio of columnA/column B. All other data is expressed as pmoles/oocyte/hr of NTP incorporated into TCA precipitable material.

Mean ± SEM 0.59 ± .44 0.25 ± .16 2.29 ± .37

and the initial rate of synthesis is the value produced by using the first time point only (Figure 4-2). Obviously this procedure will underestimate the initial rate depending on how soon the first time point was taken, however, the values in Table 4-1 show clearly that the initial rate estimated in this way is at least $2\frac{1}{2}$ times greater than the stable rate of RNA synthesis suggesting that more than 70% of the total RNA synthesized is unstable. Further, since the incorporation curves in Figure 4-2 become linear within 4 hours, the average half-life of this unstable RNA must be shorter than 4 hours. Recent studies are in agreement with these observations (Anderson & Smith, 1977). Sucrose gradient analysis of total RNA from injected stage 6 oocytes was entirely consistent with the data presented in Chapter 3 for incubated oocytes.

Incorporation into oligo (dT) bound RNA

Figure 4-3 gives the incorporation curves of radioactive GTP and ATP into oligo (dT) bound RNA by stage 6 oocytes prepared by the modified oligo (dT)-cellulose procedure outlined in materials and methods, section (h). The curves presented for 4 different females all show a definite change of slope between 20 and 40 hours of incubation although saturation may not take place. This suggests a mixture of stable and unstable RNA components with the latter predominating. It was considered important, in view of the results of Rosbash & Ford (1974) which showed that the amount of poly(U) binding activity (poly(A)$^+$RNA) remained constant from early oogenesis until ovulation, to demonstrate whether or not there was a stable component present in the newly synthesized oligo(dT) bound RNA.

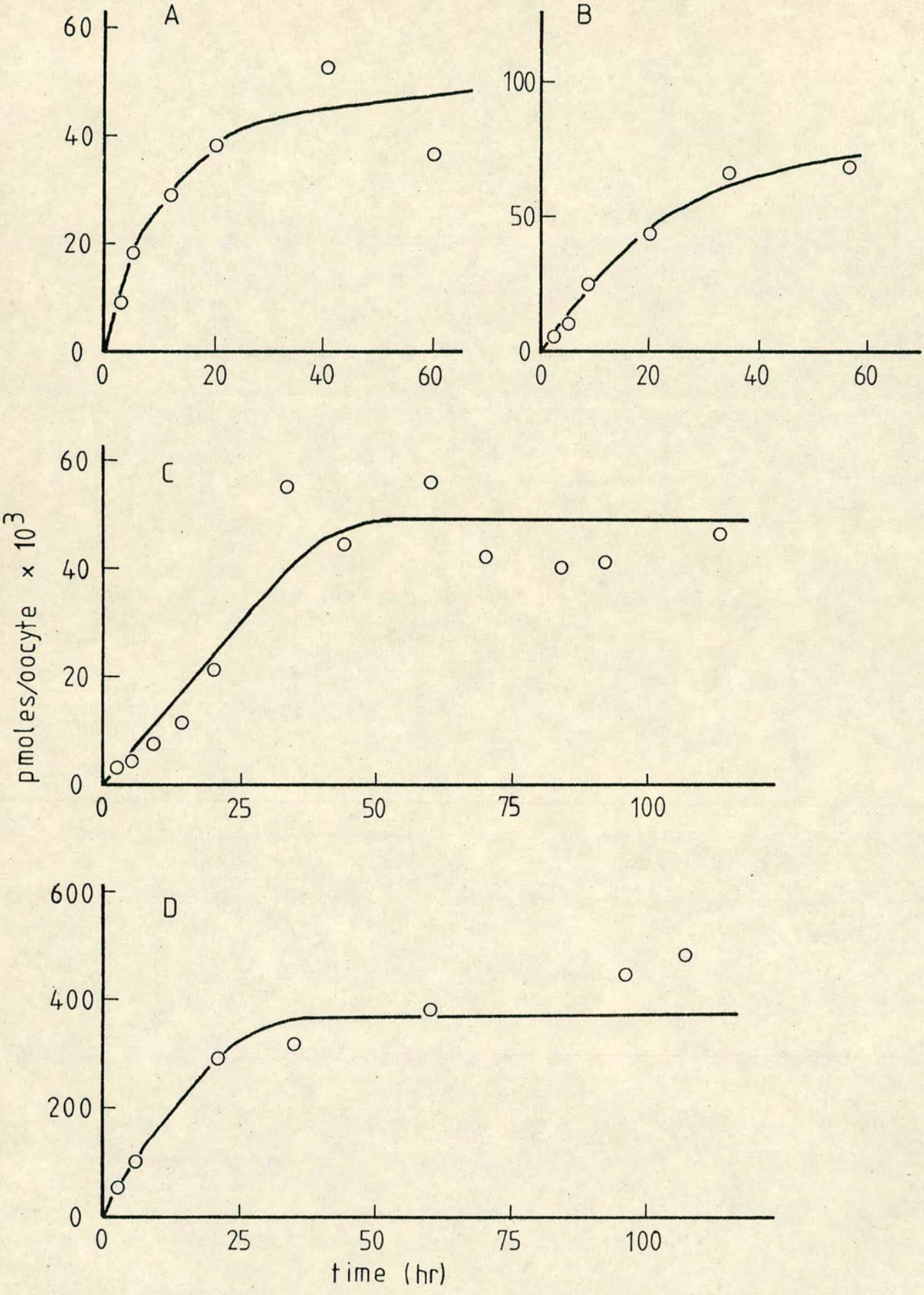

A
B
C
D
pmoles/oocyte × 10³
time (hr)
0
20
40
60
100
25
50
75
200
400
600

Figure 4-3 Incorporation into oligo (dT) bound RNA in microinjected stage 6 oocytes

Oligo (dT) bound RNA was prepared from total RNA obtained in experiments of the type described in Figure 4-1 by using the method outlined in section (h) of materials and methods. Samples of oligo (dT) bound RNA were precipitated with TCA and the radioactivity determined as described in materials and methods section (m). The cpm/oocyte incorporated were converted to pmoles/oocyte by estimating the initial specific radioactivity of the injected NTP as explained in Figure 4-2. Over 95% of the radioactivity was recovered after oligo(dT)-cellulose chromatography.

(A) Incorporation of ^{3}H-GTP

(B) Incorporation of ^{3}H-ATP

(C) Incorporation of ^{3}H-GTP (Figure 4-2D)

(D) Incorporation of ^{32}P-GTP (Figure 4-2C)

Therefore, in view of the possibility of rRNA contamination (Chapter 3, section (e)), samples of oligo (dT) bound RNA were further analysed.

Sucrose gradient analysis showed that the degree of contamination of the oligo (dT) bound RNA by rRNA increased with the time of incubation but often the data was not good enough to allow an accurate estimate of the amount of contamination. The higher counting efficiency of ^{32}P relative to ^{3}H facilitated the analysis of oligo (dT) bound RNA on SDS acrylamide gels. The results of this procedure are given in Figure 4-4. Clearly the amount of rRNA present in the samples increases with the time of incubation. Using the method of Girard et al. (1965) the percentage of rRNA contamination has been estimated in these gels and used to apply a correction to the original incorporation curve of Figure 4-3D. In Figure 4-5 this correction has been applied with the result that the incorporation curve now clearly saturates after about 20 hours. Therefore within the limits of this assay no stable component of oligo (dT) bound RNA has been observed and this whole class of RNA appears to turnover with a single half-life of about 5-10 hours. It is estimated that a stable component equal to 1/10 of the initial rate of oligo (dT) bound RNA synthesis would have been detected.

Using 4 day old ovary a similar saturation curve was obtained for oligo (dT) bound RNA synthesis. This argues that the saturation observed is not due to the exhaustion of some factor specifically required for oligo (dT) bound RNA synthesis. Nor can it be due to a systematic reduction in the recovery of the oligo (dT) bound RNA from the oligo (dT)-cellulose column. Firstly the data in Table 4-2 shows that the percentage of ^{3}H-labelled previtellogenic ovary total

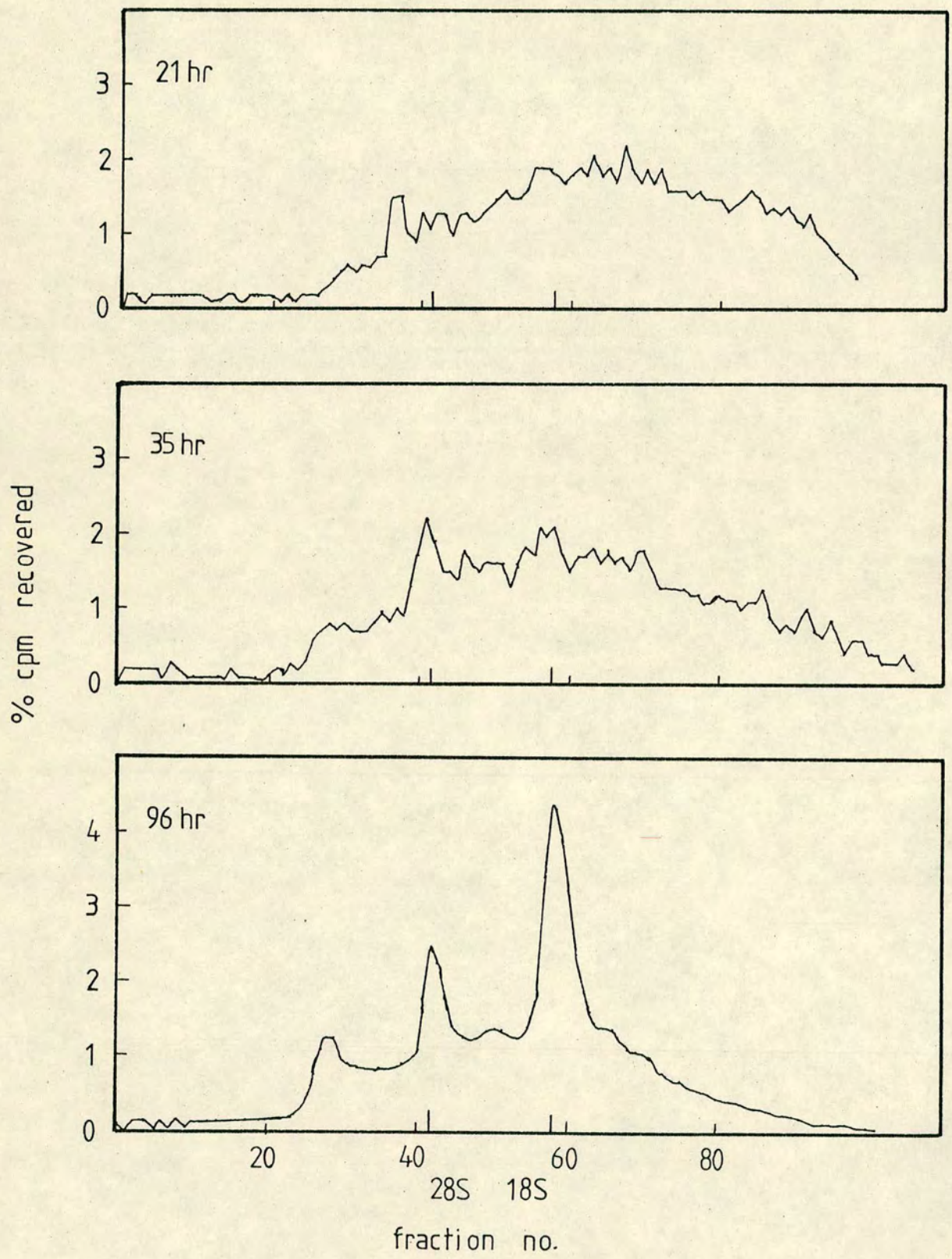

21 hr
35 hr
96 hr
% cpm recovered
0
1
2
3
4
20
40
60
80
28S
18S
fraction no.

Figure 4-4 Analysis of ^{32}P-GTP labelled oligo (dT) bound RNA on SDS/acrylamide gels

Samples of oligo (dT) bound RNA prepared from the experiment described in Figure 4-2C and Figure 4-3D were analysed on 2.3% SDS/acrylamide gels as described in materials and methods section (1). The gels were scanned at 260 nm to determine the positions of the 18S and 28S carrier RNA and then sliced into 1 mm slices and the radioactivity determined as described in section (1) of materials and methods. Data is expressed as the percentage per fraction of the total cpm recovered which was over 95% of that loaded.

21 hours, 6,200 cpm loaded; 35 hours, 6,800 cpm loaded;
96 hours, 16,500 cpm loaded.

The rRNA contamination was estimated using the method of Girard et al. (1965) and the purity of the samples was estimated to be

21 hours 96% heterogeneous RNA
35 hours 92% heterogeneous RNA
96 hours 64% heterogeneous RNA

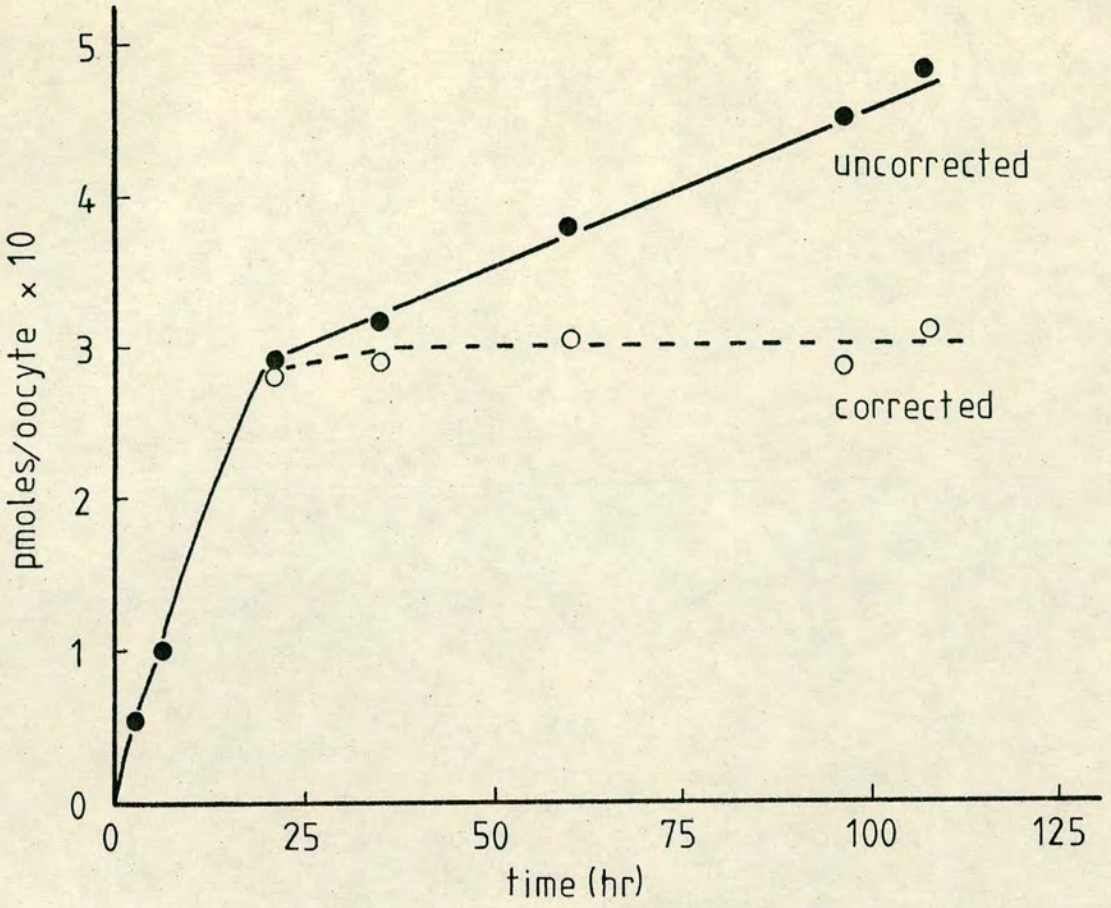


Figure 4-5 Correction to oligo (dT)$^{-}$ bound RNA incorporation curve

The incorporation curve of Figure 4-3D is reproduced here (uncorrected). Samples of oligo (dT) bound RNA were analysed on SDS/acrylamide gels to determine the degree of rRNA contamination as shown in Figure 4-4. The values for the purity of the oligo (dT) bound RNA have been used to correct the original curve.

21 hours, 96% pure;
60 hours, 80% pure;
107 hours, 65% pure.
35 hours, 92% pure;
96 hours, 64% pure;

Table 4-2 Recovery of radioactive RNA after oligo (dT)-cellulose chromatography

Sample Time Point	Load		Void		Bound	
	^{3}H cpm (%)	^{32}P cpm (%)	^{3}H cpm (%)	^{32}P cpm (%)	^{3}H cpm (%)	^{32}P cpm (%)
Control	95.0	-	92.4	-	4.5	-
Zero Time	96.1	100	94.1	100	4.4	-
$2\frac{1}{2}$	97.3	100	93.7	97.6	4.9	1.4
6	96.5	100	91.6	97.3	4.2	1.5
21	96.8	100	92.7	96.8	4.9	2.3
35	96.1	100	92.9	97.3	4.9	1.8
60	95.9	100	91.8	97.1	4.6	1.7
96	96.2	100	92.4	97.0	4.5	1.0
107	95.3	100	91.7	98.1	4.2	1.0

In the microinjection experiment described in Figure 4-4, during homogenization of the ^{32}P-ATP labelled oocytes, 140,000 cpm of ^{3}H-labelled total RNA from previtellogenic ovary was added to each sample and the RNA extracted as described in materials and methods section (g). Oligo (dT)-cellulose chromatography was carried out as described in materials and methods section (h) and samples of total RNA, oligo(dT) void RNA and oligo (dT) bound RNA were precipitated with TCA and the radioactivity determined as described in materials and methods section (m). The recovered cpm are expressed as a percentage of the 140,000 cpm added at homogenization in the case of ^{3}H and of the total cpm loaded onto the oligo (dT)-cellulose column in the case of ^{32}P.

RNA added during RNA extraction which binds to oligo (dT)-cellulose in the different time points is quite constant. Secondly, in a recent report (Cabada et al., 1977) a class of poly(A)$^+$RNA with a very short poly(A) sequence (poly(A)$_S$ RNA) was observed to accumulate during oogenesis in Xenopus laevis. Since the stringent conditions employed here to isolate poly(A)$^+$RNA would not isolate this poly(A)$_S$ RNA it is possible that the observed turnover could be accounted for by the production of poly(A)$_S$ RNA from the poly(A)$^+$RNA initially synthesized. This is considered unlikely for 2 reasons. Firstly, since attempts were made to isolate a newly labelled poly(A)$_S$ RNA fraction using the conditions of Cabada et al. (1977) without success and secondly poly(A)$_S$ RNA appears not to be present in all Xenopus laevis females and was absent in the batch of animals used in this investigation (Darnbrough, personal communication).

Table 4-3 compiles data from several experiments on the relative rates of oligo (dT) bound RNA synthesis and stable RNA synthesis and also on the steady state amount of oligo (dT) bound RNA accumulated. From this table it is seen that the steady state amount of oligo (dT) bound RNA synthesized is about 0.2-1.0% of the 30-40 ng of poly(A)$^+$RNA present in stage 6 oocytes (Rosbash & Ford, 1974). It is therefore clear that all of the stored oocyte pool of poly(A)$^+$RNA does not turnover with this 5-10 hour half-life. Since none of the newly synthesized poly(A)$^+$RNA appears to be stable, it may be that the newly synthesized oligo(dT) bound RNA detected in these experiments is a different class of poly(A)$^+$RNA from the stored pool of transcripts in stage 6 oocytes. Table 4-3 column D expresses the initial rate of oligo (dT) bound RNA synthesis as a percentage of the stable rate of total RNA synthesis. In order to obtain a maximum estimate for the percentage of the initial rate of total RNA synthesis that the initial

Table 4-3 Comparison of rates of incorporation of injected NTP into stable RNA and oligo (dT) bound RNA

		A	B	C	D	E
Exp.	Label	Total RNA Stable rate of incorpn.	Oligo (dT) bound RNA Initial rate of incorpn.	Oligo (dT) bound RNA Steady state incorpn.	Initial Rate of oligo(dT) incorpn. as a percentage of stable rate	Steady state amount of oligo (dT) bound RNA divided by stable rate
		pmoles/oo/hr	pmoles/oo/hr	pmoles/oo	%	
1	^{3}H-ATP	0.060	0.0020	0.090	3.3	1.50
2	^{3}H-GTP	0.110	0.0039	0.050	3.5	0.45
3	^{3}H-GTP	0.085	0.0015	0.055	1.8	0.65
4	^{32}P-GTP	0.400	0.0225	0.350	5.6	0.88
5	^{3}H-GTP	0.425	0.0170	0.138	4.0	0.32
6	^{32}P-ATP	0.230	0.0430	0.250	18.7	1.09
7a	^{3}H-GTP	0.180	0.0075	0.055	4.2	0.31
7b	^{32}P-ATP	0.070	0.0210	0.085	30.0	1.20
Average:						
Exps. 1-4		0.164 (209)	0.0075 (10)	0.136 (174)	3.6	0.87
Exps. 5-7		0.226 (289)	0.0221 (28)	0.132 (169)	14.2	0.73

For experiments 1-4 the data is compiled from several experiments of the type described in Figure 4-1 and 4-3. The stable rate of incorporation into total RNA (column A) corresponds to the linear part of the curves of the type in Figure 4-1. The initial rate of incorporation into oligo (dT) bound RNA (column B) is the rate to the first time point in curves of the type in Figure 4-3 and the steady state incorporation (column C) corresponds to the plateau in these curves. Data is expressed as pmoles of NTP incorporated per oocyte/hour into TCA precipitable material in columns A and B and as pmoles/oocyte in column C. In columns D and E the data is normalized by expressing as a percentage or fraction of the stable rate of incorporation. The data for experiments 5-7 are compiled from several experiments of the type in Figures 4-6 and 4-8, by summing the incorporation of NTP into RNA in germinal vesicle and cytoplasm samples. Experiment 7 was a double label experiment in which ^{32}P-ATP and ^{3}H-GTP were simultaneously injected into the same oocyte. The average values for experiments 1-4 and 5-7 are recorded separately and the number in brackets represents the rate of RNA synthesis in pg/oocyte/hour (columns A and B) or the amount accumulated in pg/oocyte (column C) calculated as described in materials and methods section (p).

rate of oligo (dT) bound RNA synthesis represents these figures should be divided by at least 2.5 (see Table 4-1). The data in Table 4-3 produces a maximum value of about 9% and the average for the 8 experiments is less than 4%. This suggests that synthesis of this class of poly(A)$^+$RNA represents only a small fraction of the oocyte's total RNA synthetic capacity. In column E of Table 4-3 the steady state amount of oligo (dT) bound RNA accumulated is normalised by dividing by the stable rate of total RNA synthesis. Comparison of these values shows reasonable agreement in the 8 experiments which suggests that in oocytes from different females a constant proportion of the total RNA synthetic capacity is directed toward synthesis of this oligo (dT) bound RNA. There is no apparent difference between these values for experiments 1-4 and those for experiments 5-8. In the latter group of experiments RNA was prepared from isolated germinal vesicles and cytoplasms which suggests no selective loss of oligo (dT) bound RNA relative to total RNA when the RNA is prepared from oocyte cell fractions. The importance of this observation will be discussed later.

(c) Cellular distribution of newly synthesized RNA

Using the modified germinal vesicle isolation procedure outlined in materials and methods, section (f) the cellular distribution of newly synthesized RNA as a function of time after microinjection of radioactive GTP or ATP was investigated.

Kinetics of synthesis and cellular distribution of total RNA

In Figure 4-6A and B the results of two such experiments are shown for total RNA synthesis. In both figures essentially the same kinetics of incorporation are seen using either radioactive GTP or

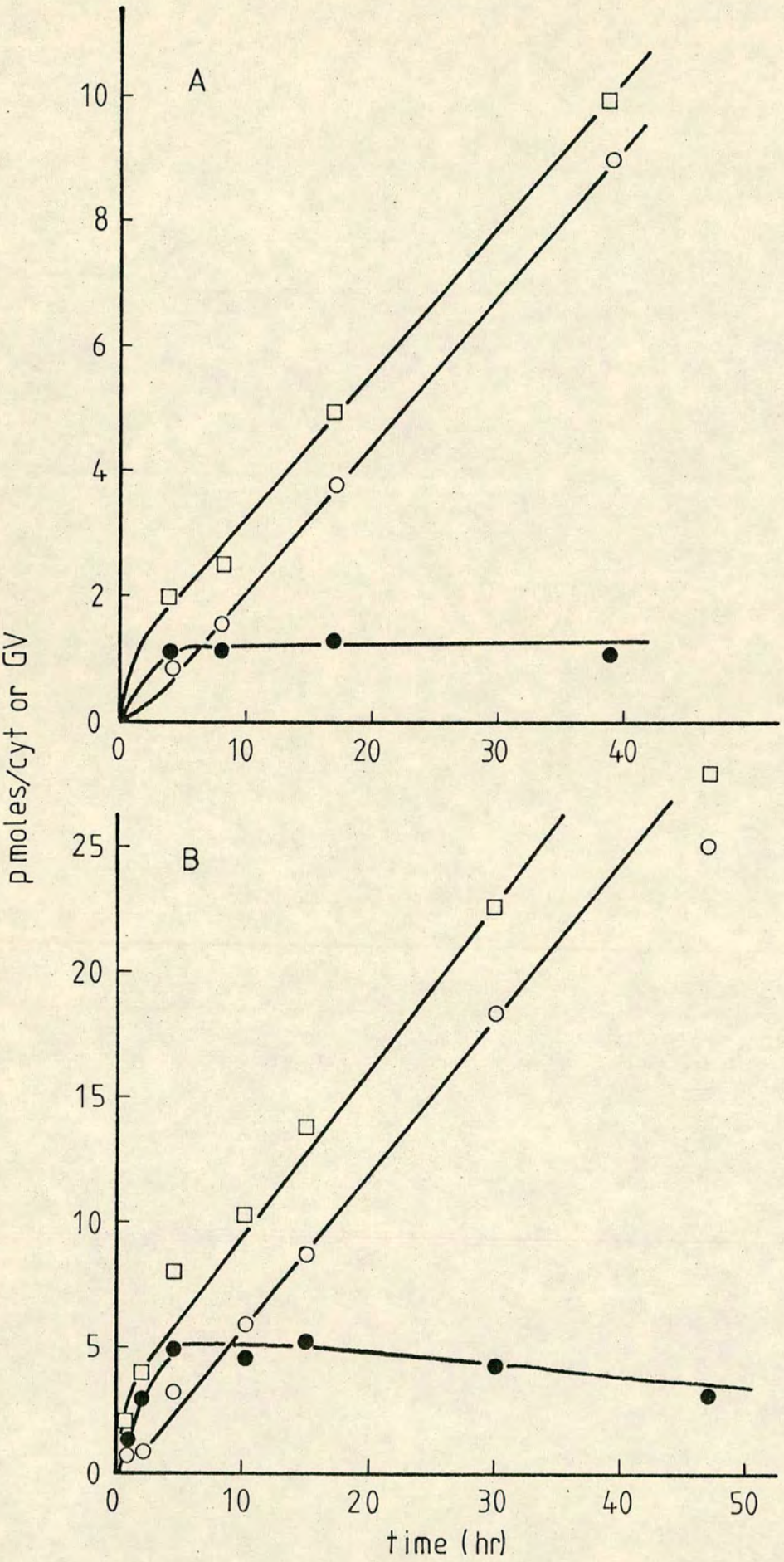

A
B
pmoles/cyt or GV
time (hr)
0
2
4
6
8
10
5
15
20
25
30
40
50

Figure 4-6 Incorporation of NTP into total RNA from germinal vesicles and cytoplasms of microinjected stage 6 oocytes

In each experiment 2-300 stage 6 oocytes were manually stripped from an ovary and microinjected in batches of 12 with 1 μCi of ^{3}H-GTP or ^{32}P-ATP per oocyte as detailed in materials and methods section (d). The oocytes were incubated for various times in glass bottles containing 250 μl of MBX, after which time they were washed several times with MBX and the follicle sheaths removed. The oocytes were then placed in GVIM and germinal vesicles and cytoplasms were prepared as described in materials and methods section (f), the individual germinal vesicles and cytoplasms being transferred as rapidly as possible to separate homogenizers containing 2 ml of modified Kirby buffer. A small volume of each total homogenate was dried onto a GF/C filter, and samples of the incubation medium were similarly treated in order to estimate the initial NTP specific radioactivity as described in Figure 4-2. RNA was extracted from the remainder of the homogenate as detailed in section (g) of materials and methods and samples of total RNA were precipitated with TCA and the radioactivity determined (section (m) of materials and methods). The cpm incorporated in both fractions were converted to pmoles incorporated as explained in Figure 4-2 and incorporation at zero time has been subtracted from the other data points.

(A) Incorporation of ^{32}P-ATP (SA about 2,000 cpm/pmole)

(●) incorporation into the germinal vesicle

(○) incorporation into the cytoplasm

(□) represents the sum of the other two curves.

(B) Incorporation of ^{3}H-GTP (SA about 900 cpm/pmole)

(●) incorporation into the germinal vesicle

(○) incorporation into the cytoplasm

(□) represents the sum of these two curves.

ATP as the injected precursor and the following observations can be made:

(1) The sum of the curves for the germinal vesicle and the cytoplasm generates a curve which is remarkably consistent with those presented earlier for the intact oocyte and therefore shows that the cell fractionation procedure used here is valid.

(2) The stable RNA made by the oocyte accumulates in the cytoplasm at a constant rate.

(3) By extrapolation there appears to be a lag of 1-2 hours before the appearance of labelled RNA in the cytoplasm and this must represent the sum of the times taken for transcription, processing and transport of the stable RNA.

(4) No significant amount of unstable RNA is detectable in the cytoplasm.

(5) The average half-life of the nuclear RNA species must be about 2-4 hours if saturation takes place within 8 hours.

Table 4-4 lists the rates of synthesis of various RNA classes and also the steady state amount of total RNA in the nucleus in a number of experiments. Dividing the steady state amount of labelled total RNA in the nucleus by the stable rate of total RNA synthesis generates a remarkably constant value for a number of experiments (columnD). This value must be related to the time taken to achieve the steady state incorporation observed in the germinal vesicle. Consequently the average half-life of nuclear RNA must be very constant in different animals.

Table 4-4 also lists the initial rate of total RNA synthesis in the germinal vesicle (as measured to the first time point, column E). Dividing this by the initial rate of total RNA synthesis in the whole

Table 4-4 Comparison of rates of incorporation of injected NTP into stable RNA and germinal vesicle RNA

Exp.	Label	A Total RNA Initial rate of incorpn. pmoles/ oo/hr	B Total RNA Stable rate of incorpn. pmoles/ oo/hr	C GV Total RNA steady state amount pmoles/GV	D Col. C / Col. B	E GV Total RNA Initial rate of incorpn. pmoles/GV/hr	F Column E as a % of Column A %	G First Time Point hrs
1	^{32}P-ATP	0.50	0.23	1.10	4.8	0.28	54	4
2	^{3}H-GTP	1.25	0.43	3.25	7.4	0.99	79	1
3	^{32}P-GTP	-	0.40	2.40	6.0	-	-	-
4	^{3}H-GTP	0.64	0.18	1.46	8.1	0.47	74	4
5	^{3}H-GTP	-	0.48	4.40	9.1	-	-	-
6	^{32}P-ATP	0.24	0.06	0.48	8.0	0.11	44	4
Average		0.65 (842)	0.36 (455)	2.61 (3351)	-	0.46 (592)	-	-

Data compiled from experiments of the type described in Figures 4-6 and 4-8. Incorporation into total RNA is the sum of the incorporations into the germinal vesicle and cytoplasm. Initial rates of synthesis are estimated to the first time point (columns A and E). The stable rates and steady state amounts were determined as described in Table 4-3. In column D the data for the steady state incorporation into total RNA in the germinal vesicle (column C) has been normalized by dividing by the stable rate of incorporation (column B) and the value generated probably relates to the time taken to reach the steady state in the germinal vesicle. Column F expresses the initial rate of incorporation into germinal vesicle RNA as a percentage of the initial rate of incorporation into total RNA and column G shows the first time point taken in the various experiments. All data expressed in a similar manner to that of Table 4-3 including the average values and the rates and amounts of RNA shown in brackets.

0.66±.43 .30±.17 2.18±1.46 72±1.6 .46±.38 63±17

oocyte (ie the sum of nucleus and cytoplasm, column A) gives values which approach 100% (column F) depending obviously on how soon the first time point was taken. This together with the observed lag before appearance of label in cytoplasmic RNA suggests an entirely nuclear location for the synthesis of the bulk of the total RNA.

The germinal vesicle incorporation curve for radioactive GTP (Figure 4-6B) appears to decrease somewhat after long incubation times and this observation has been made occasionally in other similar experiments. It should be noted that the last cytoplasm time point is also low. A reasonable explanation for this observation is that the specific activity of the NTP had decreased significantly during this experiment, and this was probably due to higher than average leakage of injected label and/or a high rate of RNA synthesis. Normally no decrease in the rate of incorporation was observed.

Analysis of total RNA

Figure 4-7 shows sucrose gradient profiles of total nuclear RNA and total cytoplasmic RNA isolated at different times after injection of labelled NTP. Using the method of Girard et al. (1965) to estimate the proportion of the total RNA due to each species, the profiles of germinal vesicle total RNA show that after 2 hours of incubation peaks of radioactivity are seen at 32S (12%), 20S (14%) and 9S (8%) upon a heterogeneous background of RNA (66%). After 5 hours the size of the 32S peak has increased and the 9S and 20S peaks are less prominent against the heterogeneous RNA. This profile changes little after longer periods of incubation and the percentage of RNA in the various species when the steady state is reached is:

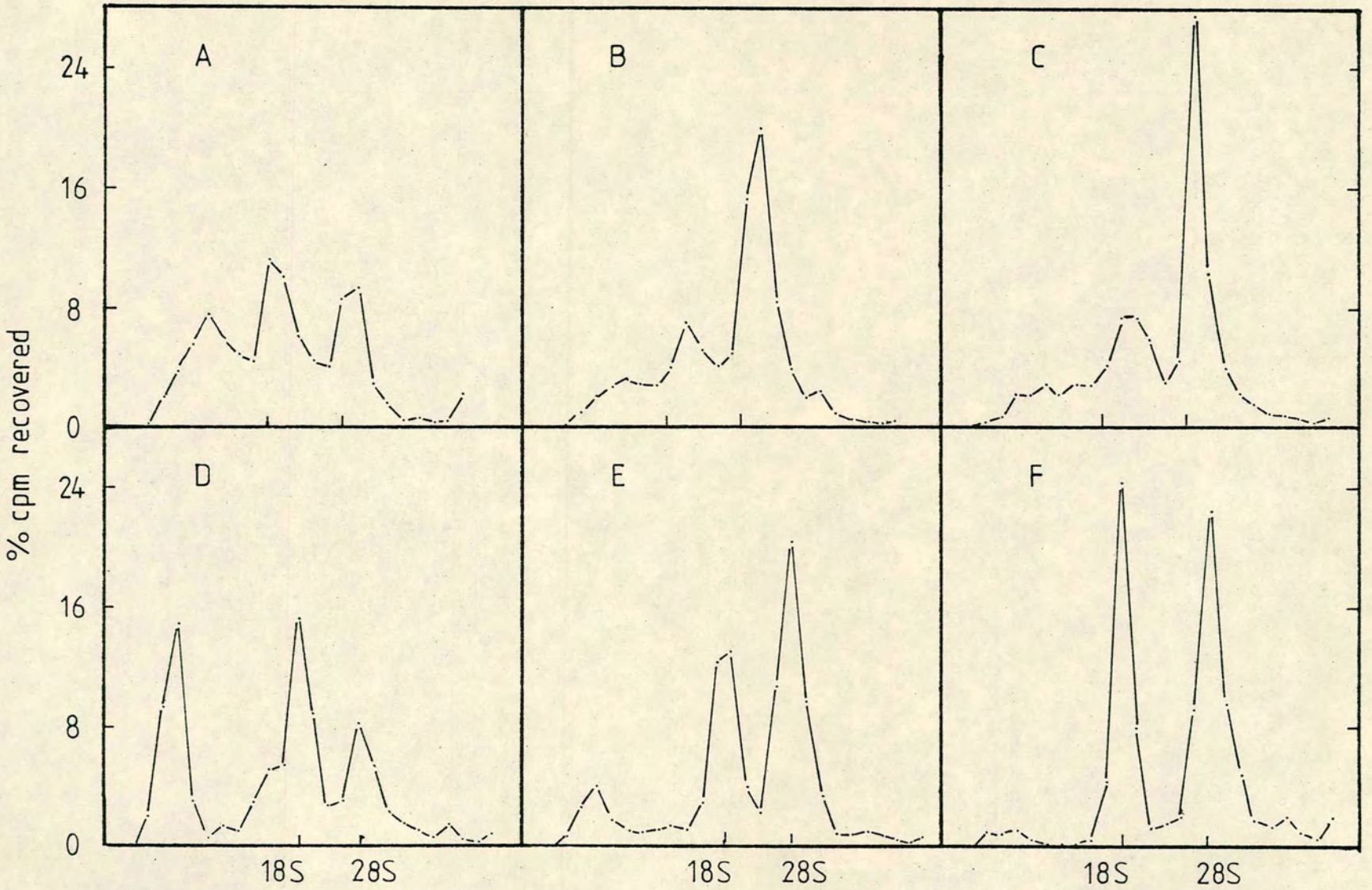

A
B
C
D
E
F
24
16
8
0
% cpm recovered
18S
28S

Figure 4-7 Sucrose gradient analysis of labelled total RNA from stage 6 oocyte germinal vesicles and cytoplasms

Samples of germinal vesicle and cytoplasm total RNA from experiments of the type described in Figure 4-6 were sedimented in 11.5 ml linear 7-30% sucrose gradients in NETS buffer at 40,000 rpm for 4½ hours at 25°C in an MSE 6 x 14 ml Ti rotor. Gradients were analysed and the radioactivity determined as described in materials and methods sections (i) and (m). Recovery of loaded cpm was over 75% and the figures show the percentage per fraction of total recovered cpm. The direction of centrifugation was from left to right and the last fraction corresponds to the pellet. The following cpm were recovered:

Germinal Vesicle total RNA

(A) Incorporation of ^{3}H-GTP for 2 hours, 1,800 cpm

(B) Incorporation of ^{3}H-GTP for 15 hours, 2,150 cpm

(C) Incorporation of ^{3}H-GTP for 26 hours, 2,100 cpm

Cytoplasm total RNA

(D) Incorporation of ^{32}P-ATP for 8 hours, 2,500 cpm

(E) Incorporation of ^{32}P-ATP for 40 hours, 4,800 cpm

(F) Incorporation of ^{3}H-GTP for 43 hours, 3,200 cpm

heterogeneous RNA about 50%; 28/32S about 38%; 20S about 10% and 9S about 2%. Since at all times 50% or more of the newly synthesized nuclear total RNA is heterogeneous in size and since it has been shown in the previous section that approximately 70% of the newly synthesized oocyte RNA turns over in the nucleus it is hard not to draw the conclusion that the majority of this unstable nuclear RNA is heterogeneous in size.

The sucrose gradient profiles of cytoplasmic RNA show that at early times (8 hours) the predominant labelled species of RNA are 18S (30%), 28S (11%), 4S/5S (28%) and heterogeneous RNA (about 31%) while after 40 hours of labelling the cytoplasmic distribution of label is 28S (41%), 18S (26%), 4S/5S (8%) and a maximum of 25% heterogeneous RNA. From these observations it should be noted that processing and/or transport of 18S and 4S/5S RNA is faster than for 28S RNA, since not until about 40 hours is the expected ratio of 0.5 for newly synthesized 18S:28S RNA approached. The amount of newly synthesized heterogeneous RNA in the cytoplasm is likely to be overestimated by this method and therefore it must be emphasised that after 40 hours of labelling the estimated value of 25% heterogeneous RNA in the cytoplasm is a maximum. If newly synthesized heterogeneous RNA is present in the cytoplasm it must be relatively stable to give the fairly constant proportions shown in Figure 4-7, although the actual amount present is likely to be less than 20% of the total labelled cytoplasm RNA.

The appearance of both the nuclear and cytoplasmic total RNA on sucrose gradients suggests that the germinal vesicle isolation procedure used results in little or no degradation of the RNA.

Cellular distribution of oligo (dT) bound RNA

In Figure 4-8 the kinetic curves of incorporation of radioactive ATP and GTP into nuclear and cytoplasmic oligo (dT) bound RNA are given. Study of these figures allows the following points to be made:

(1) The curves generated by summing the individual nucleus and cytoplasm curves are similar to those presented earlier for intact oocyte oligo (dT) bound RNA, in that they saturate (if allowance is made for rRNA contamination) after about 20 hours although, in the case of radioactive ATP incorporation, saturation may occur sooner than this.

(2) For radioactive ATP incorporation into oligo (dT) bound RNA, at saturation, cytoplasmic incorporation accounts for 60% of the total incorporation into oligo (dT) bound RNA. The curve shows similar kinetics to the total oocyte curve.

(3) Although at saturation 40% of the total ATP incorporation into oligo (dT) bound RNA is in the nucleus, this incorporation curve saturates slightly earlier than the cytoplasmic curve and consequently about 50% of the incorporation into oligo (dT) bound RNA is nuclear after only 4 hours.

(4) When labelling with GTP very little incorporation is observed into germinal vesicle oligo (dT) bound RNA, and at least 85% of the total incorporation into oligo (dT) bound RNA is found in the cytoplasm at all times.

(5) In oocytes from the same female as for ^{3}H-GTP (Figure 4-8B), radioactive ATP incorporation into oligo (dT) bound RNA after 28 hours showed an approximately equal distribution between nucleus and cytoplasm.

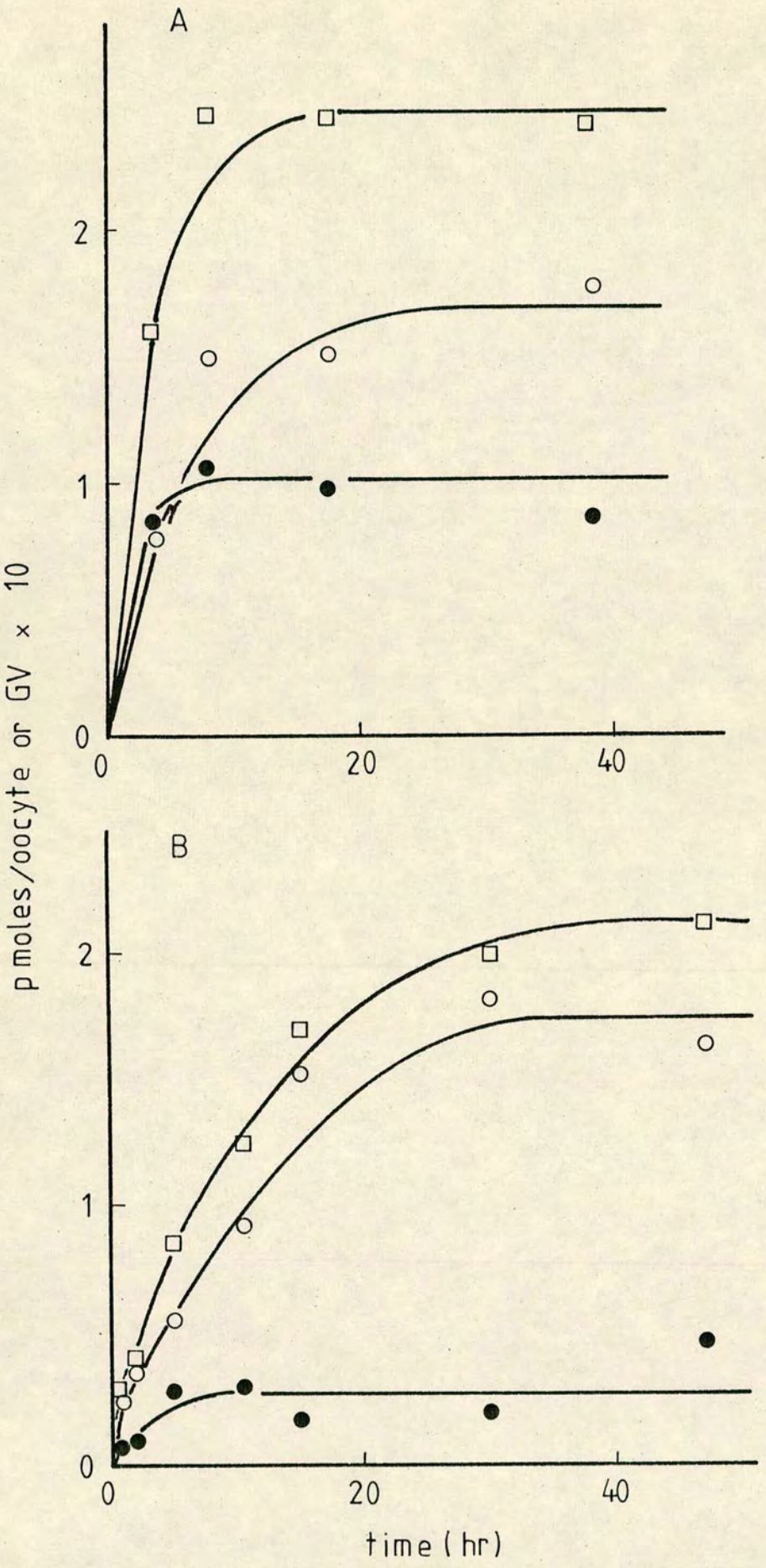

A
B
2
1
0
0
20
40
p moles/oocyte or GV × 10
time (hr)

Figure 4-8 Incorporation of NTP into oligo (dT) bound RNA from germinal vesicles and cytoplasms of stage 6 oocytes

Oligo (dT) bound RNA was prepared from samples of total RNA obtained in the experiments described in Figure 4-6 as detailed in materials and methods section (h). Samples of oligo (dT) bound RNA were precipitated with TCA and the radioactivity determined as described in section (m) of materials and methods. Recovery of loaded cpm after oligo (dT)-cellulose chromatography was over 97%. The cpm of NTP incorporated were converted to pmoles of NTP incorporated using the estimated specific radioactivity determined as in Figures 4-2 and 4-6.

(A) Incorporation of ^{32}P-ATP (SA about 2,000 cpm/pmole)

(●) incorporation into the germinal vesicle

(○) incorporation into the cytoplasm

(□) represents the sum of these two curves.

(B) Incorporation of ^{3}H-GTP (SA about 900 cpm/pmole)

(●) incorporation into the germinal vesicle

(○) incorporation into the cytoplasm

(□) represents the sum of these two curves.

Table 4-5 gives the steady state amount of labelled oligo (dT) bound RNA as a percentage of the steady state amount of labelled total RNA in the germinal vesicle in 4 experiments using ATP. It is very constant at about 7% (the equivalent value for GTP is less than 0.5%). This suggests that radioactive ATP is incorporated into a nuclear RNA species which is only slightly if at all labelled by radioactive GTP.

These observations do not give a clear cut answer as to the site of synthesis of oligo (dT) bound RNA within the oocyte. Detecting little or no nuclear oligo (dT) bound RNA labelled with GTP whereas the cytoplasmic fraction labels rapidly may mean that processing and transport of this species, if it is made in the nucleus, is sufficiently fast to keep the nuclear pool of it very small. Alternatively the apparent lack of GTP labelled nuclear oligo (dT) bound RNA at all times investigated may mean that newly synthesized oligo (dT) bound RNA is actually made in the cytoplasm in stage 6 oocytes.

The samples of oligo (dT) bound RNA prepared from nuclear and cytoplasmic fractions were analysed on sucrose gradients and typical results are given in Figure 4-9. There was insufficient incorporation of radioactive GTP into nuclear oligo (dT) bound RNA to analyse on gradients. The profiles of cytoplasmic oligo (dT) bound RNA labelled with either ATP or GTP are similar and closely resemble the profiles for the intact oocyte. Occasionally a peak of radioactivity was present in ATP labelled cytoplasmic oligo (dT) bound RNA which sedimented at about 7S. The gradient profile of ^{32}P-ATP (or ^{3}H-ATP) labelled oligo (dT) bound RNA from germinal vesicles shows a single peak of radioactivity sedimenting at about 7S, and this profile changed little after long incubation times.

Table 4-5 Incorporation of radioactive ATP into germinal vesicle RNA

		A	B	C
Exp.	Label	GV Total RNA Steady state amount pmoles/GV	Oligo (dT) bound RNA Steady state amount pmoles/GV	Column B as percentage of column A %
1	^{32}P-ATP	0.44	0.031	7.1
2	^{3}H-ATP	2.20	0.163	7.4
3	^{32}P-ATP	1.10	0.088	7.8
4	^{3}H-ATP	0.41	0.026	6.3
Average		1.03 (1330)	0.077 (98)	

Data compiled from several experiments of the type described in Figures 4-5 and 4-8 for radioactive ATP injections only. The steady state amounts of RNA accumulated are read directly from the curves and are expressed in pmoles/germinal vesicle (GV). Column C expresses the steady state amount of incorporation into oligo (dT) bound RNA in the germinal vesicle as a percentage of the incorporation into germinal vesicle total RNA. The values in brackets are amounts of RNA calculated as detailed in materials and methods section (p).

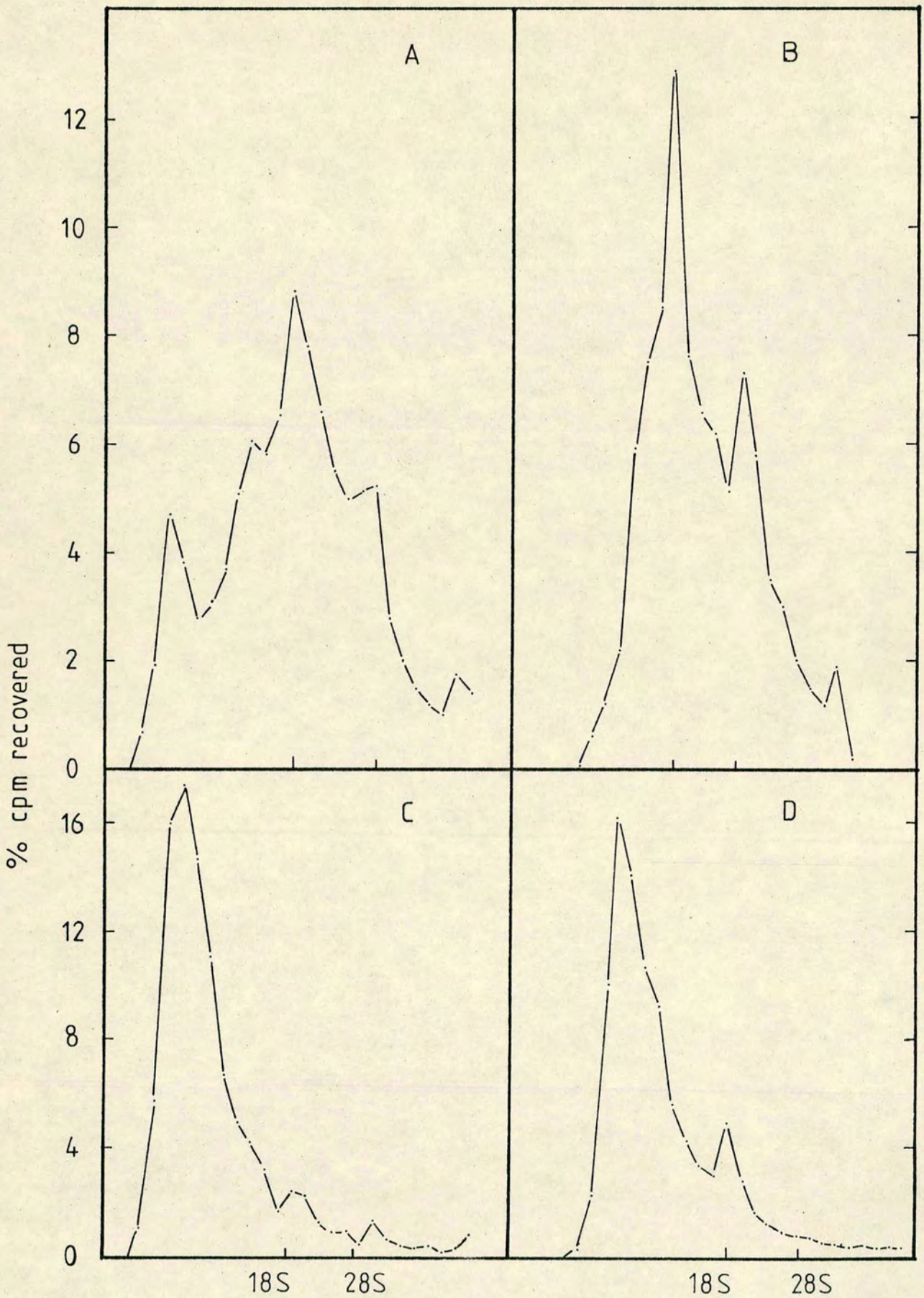

A
B
C
D
% cpm recovered
0
2
4
6
8
10
12
16
18S
28S
18S
28S

Figure 4-9 Sucrose gradient analysis of labelled oligo (dT) bound RNA from germinal vesicles and cytoplasms of stage 6 oocytes

Samples of germinal vesicle and cytoplasm oligo (dT) bound RNA from the experiments described in Figure 4-8 were sedimented in 11.5 ml 7-30% sucrose gradients in NETS exactly as described in Figure 4-7. The gradients were analysed and the radioactivity determined as described in materials and methods sections (i) and (m). Recovery of loaded cpm was 70-80% and the figures show the percentage per fraction of the total recovered cpm. The direction of centrifugation was from left to right and the last two fractions correspond to the pellet. The following cpm were recovered.

Cytoplasmic oligo (dT) bound RNA

(A) Incorporation of ^{32}P-ATP for 4 hours, 2,100 cpm

(B) Incorporation of ^{3}H-GTP for 45 hours, 2,000 cpm

Germinal Vesicle oligo (dT) bound RNA

(C) Incorporation of ^{32}P-ATP for 8 hours, 1,400 cpm

(D) Incorporation of ^{32}P-ATP for 30 hours, 3,100 cpm

These observations suggest that either there exists in stage 6 oocyte nuclei a species of A-rich RNA which sediments at about 7S or that some species of RNA is degraded to a size of about 7S which is A-rich. An experiment was performed in an attempt to distinguish between these possibilities. It was argued that if this 7S A-rich RNA is a normal component of the oocyte then it should be possible to detect a peak of radioactivity in this position on sucrose gradients of intact oocyte ATP labelled oligo (dT) bound RNA. In Figure 4-10 it is clear that unmanipulated oocytes labelled with either ATP or GTP synthesize oligo (dT) bound RNA with similar sedimentation properties. The oligo (dT) bound RNA from the germinal vesicle and to some extent the cytoplasm shows this peak at 7S when sedimented on sucrose gradients. It would seem that this 7S A-rich RNA is produced as a result of the germinal vesicle isolation procedure and is probably a degradation product. The observations that the 7S RNA is A-rich and may be a product of degradation of some larger RNA species suggest that it might consist largely of poly(A), since this molecular species is quite resistant to RNAse (Darnell et al., 1971; Edmonds et al., 1971) and an experiment was performed in order to test this hypothesis.

7S germinal vesicle poly(A)

Samples of ^{32}P-ATP labelled oligo (dT) bound RNA from stage 6 oocyte germinal vesicles and cytoplasms were sedimented on sucrose gradients and the fractions indicated in Figure 4-11 were pooled. The RNA in these samples was digested with DNAse and with RNAses A and T_1 and the digest was applied to an oligo (dT)-cellulose column. The radioactivity in the void and bound fractions from each sample was measured by Cerenkov counting and these values are also given.

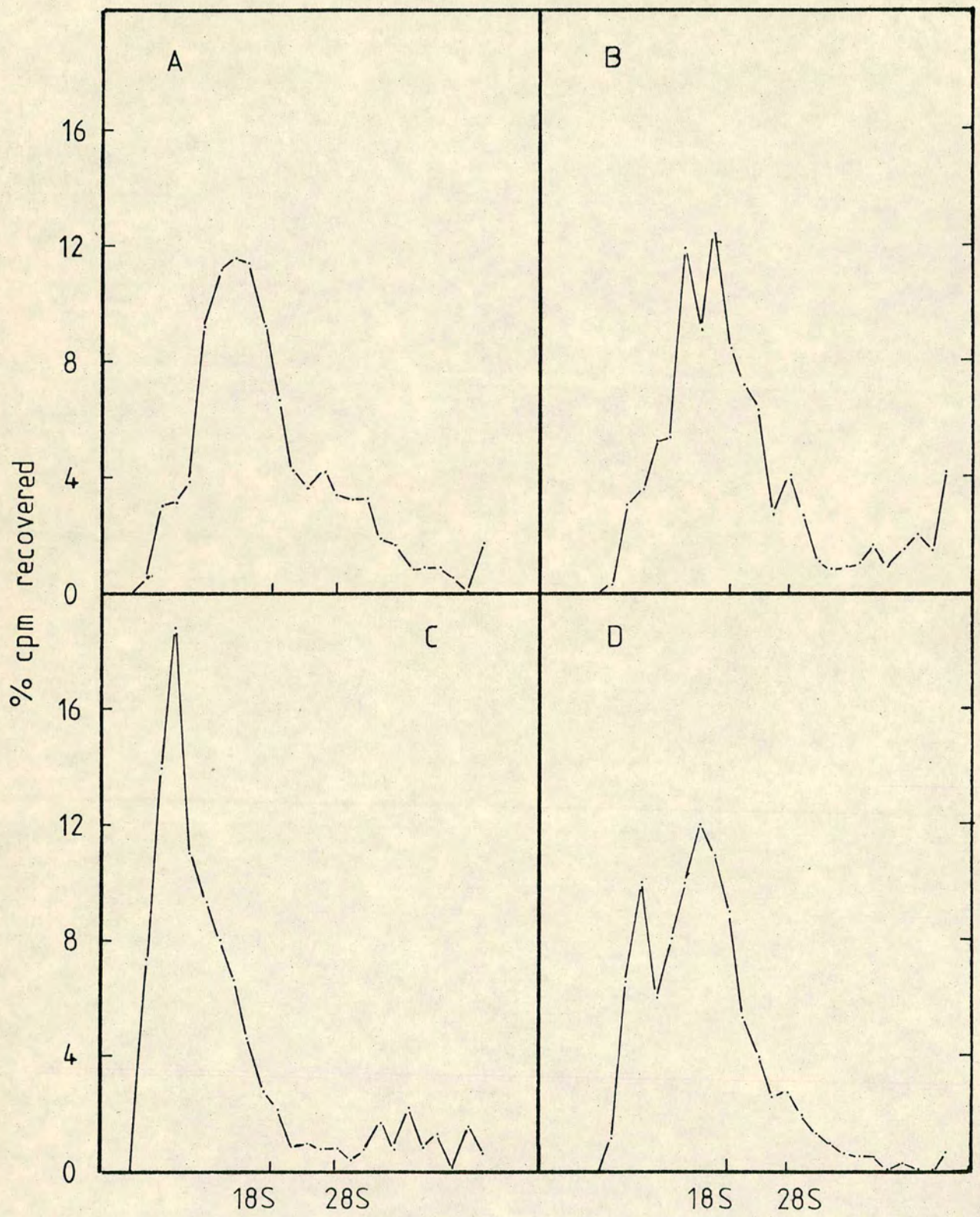

A
B
C
D
16
12
8
4
0
% cpm recovered
18S
28S
18S
28S

Figure 4-10 Sedimentation analysis of ATP and GTP labelled oligo (dT) bound RNA from intact oocytes, germinal vesicles and cytoplasms.

3 batches of 50 stage 6 oocytes were each microinjected with 1 μCi of ^{3}H-ATP and 1 batch of 50 were microinjected with 1 μCi of ^{3}H-GTP each. The injected oocytes were incubated for 24 hours in small petri dishes containing 2 ml of MBX and then washed several times and the follicle sheaths removed as detailed in materials and methods section (e). The ^{3}H-GTP injected oocytes and one batch of ^{3}H-ATP injected oocytes were homogenized immediately in 2 ml modified Kirby buffer and germinal vesicles and cytoplasms were isolated from the remaining ^{3}H-ATP injected oocytes before homogenization (materials and methods section (f)). Total RNA and then oligo (dT) bound RNA were prepared from each of the 4 samples as detailed in sections (g) and (h) of materials and methods. Samples of oligo (dT) bound RNA were sedimented in 11.5 ml linear 7-30% sucrose gradients in NETS exactly as described in Figure 4-7 and the gradients were analysed and the radioactivity determined as described in materials and methods section (i) and (m). Recovery of loaded cpm was 70-80% and the figures show the percentage per fraction of the total recovered cpm. Centrifugation was from left to right and the following cpm were recovered:

<u>Oocyte oligo (dT) bound RNA</u>

(A) Incorporation of ^{3}H-ATP, 2,000 cpm recovered

(B) Incorporation of ^{3}H-GTP, 1,700 cpm recovered

<u>Germinal vesicle oligo (dT) bound RNA</u>

(C) Incorporation of ^{3}H-ATP, 1,400 cpm recovered

<u>Cytoplasmic oligo (dT) bound RNA</u>

(D) Incorporation of ^{3}H-ATP, 1,600 cpm recovered

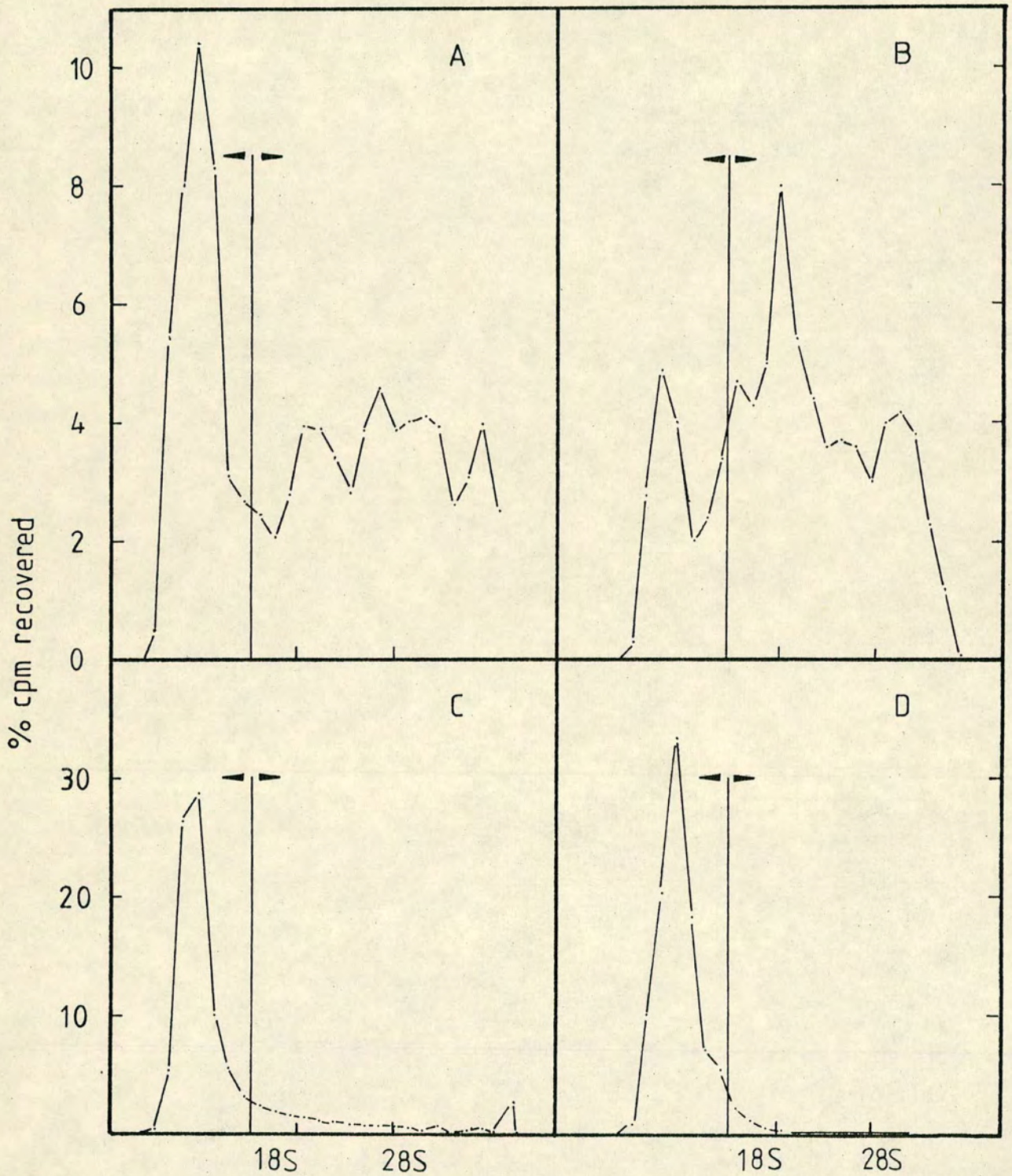

A
B
C
D
% cpm recovered
10
8
6
4
2
0
30
20
10
18S
28S
18S
28S

Figure 4-11 Sedimentation of ^{32}P-ATP labelled oligo(dT) bound RNA from germinal vesicles and cytoplasms

Samples of germinal vesicle and cytoplasmic oligo (dT) bound RNA labelled for 4 hours and 70 hours with ^{32}P-ATP, in an experiment of the type described in Figures 4-6 and 4-8 was sedimented in 11.5 ml linear 7-30% sucrose gradients in NETS at 40,000 rpm for 4½ hours at 25°C in an MSE 6 x 14 ml Ti rotor. Gradients were analysed and 1/10 of each fraction was precipitated with TCA and the radioactivity determined as detailed in materials and methods sections (i) and (m). Fractions 1-7 and 8-23 of each of the 4 gradients were pooled separately and the RNA precipitated with ethanol. The redissolved RNA was then digested with RNAse to prepare poly(A) as described in materials and methods section (j) and the radioactivity present in the oligo (dT)-cellulose void and bound fractions was determined by Cerenkov counting (see below). The RNA in the bound fractions was then precipitated with ethanol using E. coli tRNA as carrier.

(A) and (B) Cytoplasmic oligo (dT) bound RNA

(C) and (D) Germinal vesicle oligo (dT) bound RNA

Time point	Sample	Oligo (dT) void		Oligo (dT) bound	
		cpm	% total recovered	cpm	% total recovered
4 hours	GV_{1-7}	569	8%	6,153	92%
	GV_{8-23}	780	26%	2,225	74%
	Cyt_{1-7}	1,051	15%	5,818	85%
	Cyt_{8-23}	8,428	63%	5,149	37%
70 hours	GV_{1-7}	287	18%	1,348	82%
	GV_{8-23}	-	-	-	-
	Cyt_{1-7}	786	31%	1,758	69%
	Cyt_{8-23}	5,966	72%	2,364	28%

Clearly over 70% of the material sedimenting at about 7S in germinal vesicle and cytoplasm gradients, at both times, is poly(A) as indicated by RNAse T_1 resistance, affinity for oligo (dT)-cellulose and labelling with ATP. RNA sedimenting faster than 7S in the cytoplasm gradients contains less poly(A), whereas 75% of the incorporation in the heavier fractions of the germinal vesicle gradient appears to be in poly(A). These oligo (dT) bound samples of RNA were then run on 10% SDS/acrylamide gels in order to size the newly synthesized poly(A) in each of the pooled fractions and the results are given in Figure 4-12. At both 4 hours and 70 hours the broad size distribution of nuclear poly(A) (30-150 A residues) is quite different from the more homogeneous cytoplasmic poly(A) size distribution (peak at 60 A residues). There was no detectable difference between the poly(A) size distributions obtained from different regions of the sucrose gradients in either nuclear or cytoplasmic samples.

The values of TCA precipitable cpm shown in Figure 4-12 can be converted into numbers of molecules of poly(A) and the basis for this calculation is outlined in materials and methods, section (p). Figure 4-13 presents the results of this procedure on the data in Figure 4-12. At 4 hours and at 70 hours the germinal vesicle poly(A) comprises heterogeneous sized molecules with 2 peaks corresponding to about 60 and 30 A residues. The cytoplasm samples are predominently homogeneous molecules of about 60 A residues long. At 4 hours about $\frac{2}{3}$ of the total labelled molecules are present in the nuclear samples whereas at 70 hours the majority of the newly labelled poly(A) molecules are in the cytoplasm. This must reflect the fact noted earlier that at 4 hours the steady state amount of cytoplasmic

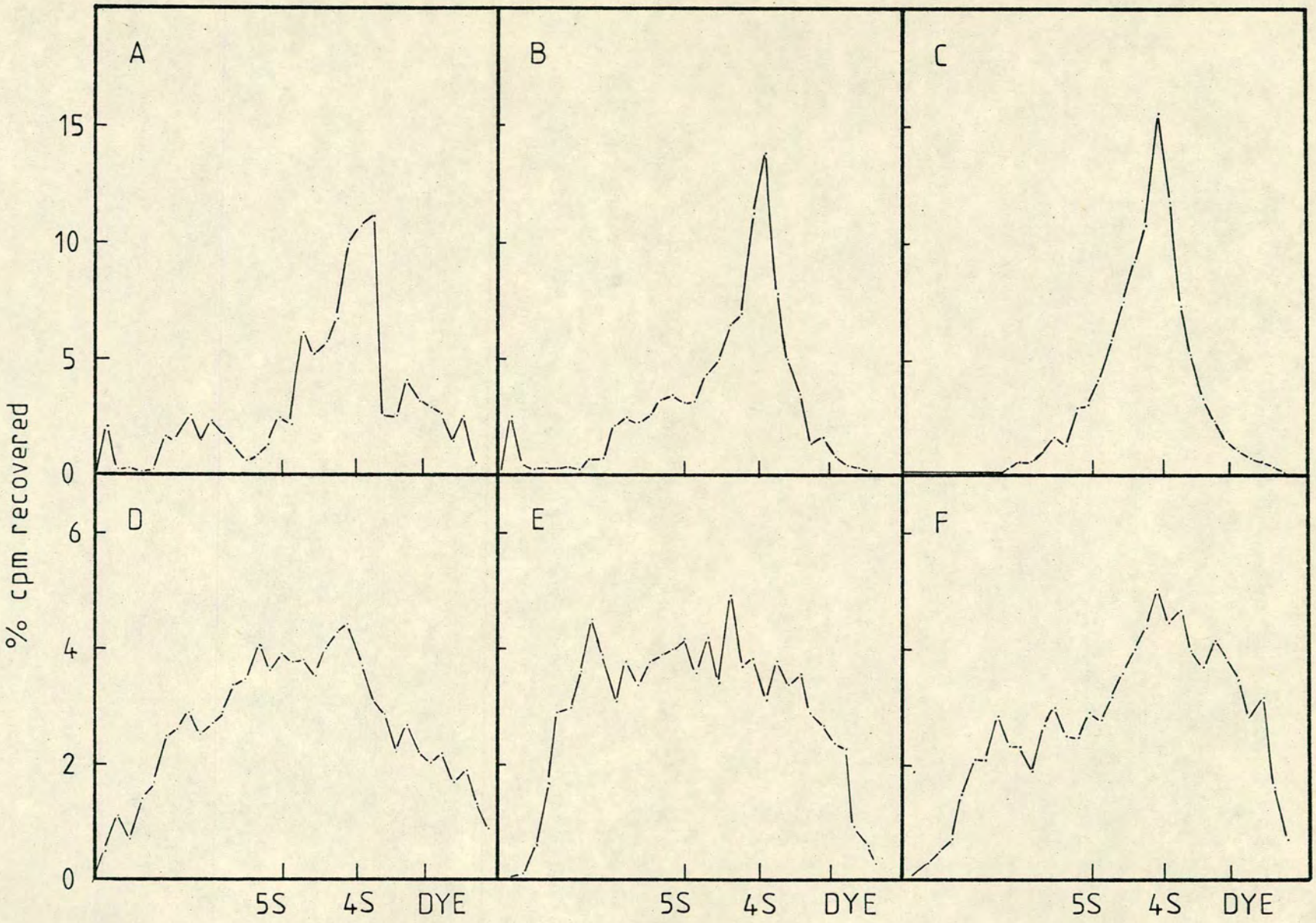

A
B
C
D
E
F
15
10
5
0
6
4
2
0
% cpm recovered
5S
4S
DYE
5S
4S
DYE
5S
4S
DYE

Figure 4-12 Poly(A) sizes of newly synthesized oligo (dT) bound RNA from stage 6 oocyte germinal vesicles and cytoplasms

The poly(A) from 6 of the 8 pooled gradient fractions of oligo(dT) bound RNA from the experiment described in Figure 4-11 were analysed on 10% SDS/acrylamide gels as described in materials and methods section (l). The gels were sliced into 1 mm slices and the radioactive poly(A) eluted from 2 adjacent slices. The poly(A) was precipitated with TCA and the radioactivity determined as detailed in materials and methods sections (l) and (m). Data is expressed as the percentage per fraction of the total recovered cpm and over 65% of the loaded cpm (Figure 4-11) were recovered. Two parallel gels were run and scanned to determine the relative positions of 5S, 4S and bromophenol blue dye which have been shown to migrate to positions corresponding to 82, 57 and 38 A residues respectively (Cabada et al., 1977).

Cytoplasmic newly synthesized poly(A)

(A) fractions 1-7 labelled for 4 hours

(B) fractions 8-23 labelled for 4 hours

(C) fractions 8-23 labelled for 70 hours

Germinal vesicle newly synthesized poly(A)

(D) fractions 1-7 labelled for 4 hours

(E) fractions 8-23 labelled for 4 hours

(F) fractions 1-7 labelled for 70 hours

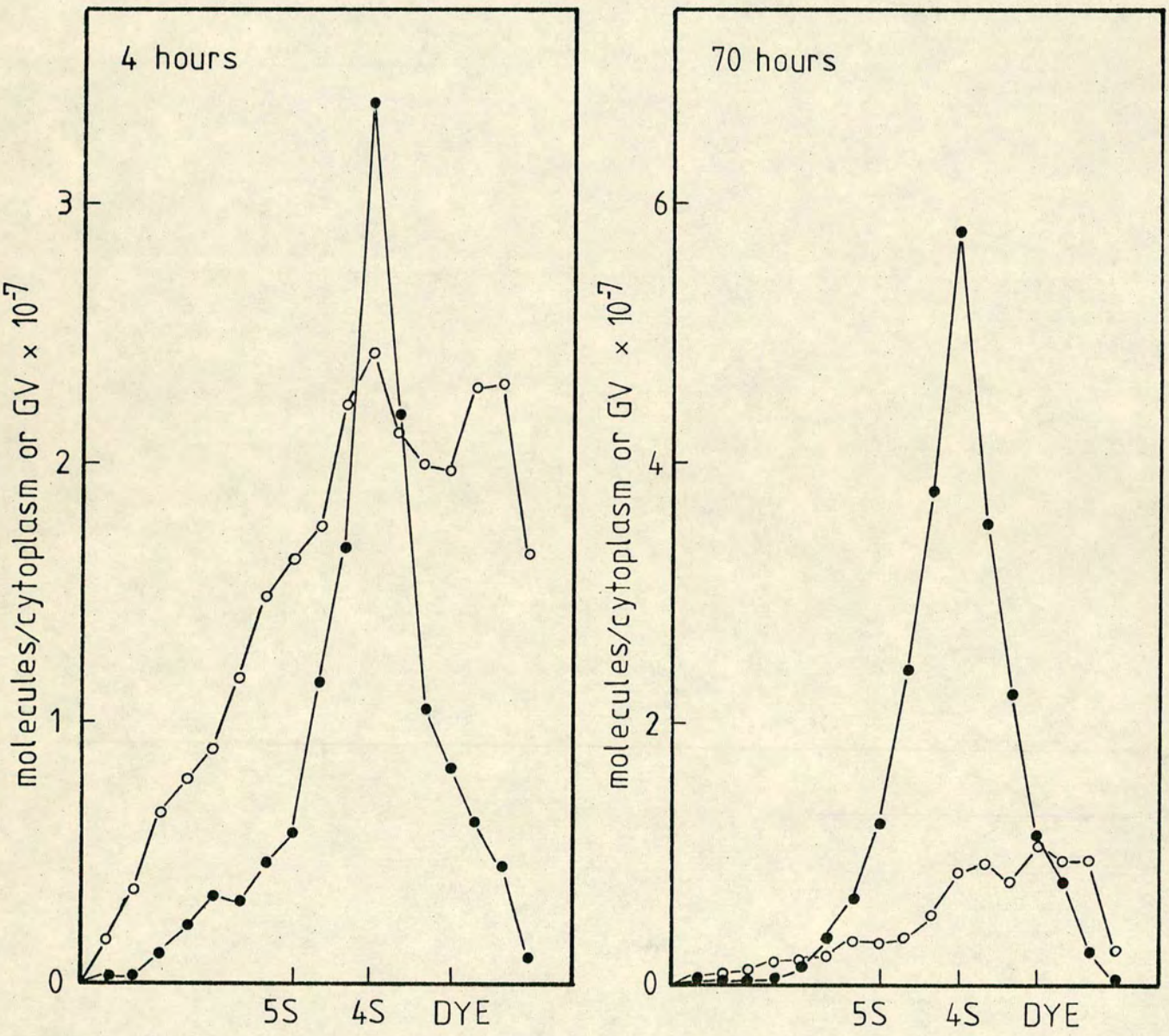

4 hours
70 hours
molecules/cytoplasm or GV × 10⁻⁷
0
1
2
3
4
6
5S
4S
DYE

Figure 4-13 Number of newly synthesized poly(A) molecules per germinal vesicle or cytoplasm

The data of Figure 4-12 was converted to the number of molecules of poly(A) per germinal vesicle or cytoplasm as detailed in materials and methods section (p). The specific radioactivity of the ^{32}P-ATP in this experiment was estimated to be 3,200 cpm/pmole using the method outlined in Figure 4-2. At both time points the molecules in fractions 1-7 and 8-23 have been combined. Since no data was obtained for the 70 hour germinal vesicle fractions 8-23 the contribution of the germinal vesicle poly(A) to the total number of molecules may be underestimated at this time point.

(o) Number of molecules of newly synthesized poly(A) per germinal vesicle.

(•) Number of molecules of newly synthesized poly(A) per cytoplasm.

poly(A)$^+$RNA has not yet been synthesized. The total number of newly labelled molecules is 3-4 x 10^8 and this represents about 0.5-1.0% of the total number of poly(A) molecules present in a stage 6 oocyte as measured by poly (U) hybridization (Cabada et al., 1977). Further if 10% of the total oocyte poly (U) binding activity is in the germinal vesicle (Turner, unpublished) then the percentage of the total steady state germinal vesicle poly(A) which becomes labelled in 4 hours is around 5%. The corresponding value for the cytoplasm is less than 0.5%

The observations outlined in this section do not allow a firm conclusion to be drawn as to the cellular site of synthesis of oligo (dT) bound RNA, therefore attempts were made to resolve this problem by using a different approach and these are presented in the next section.

(d) RNA synthesis in enucleated stage 6 oocytes

The large size of the germinal vesicle in stage 6 Xenopus laevis oocytes enables enucleation to be performed fairly easily. The method used here is essentially that of Gall (1966) and is described in materials and methods, section (f). Enucleation should permit the direct comparison of the products of cytoplasmic, or mitochondrial, synthetic activity with those of the nucleus, or the total oocyte. Since, during oogenesis mitochondria accumulate in the cytoplasm of oocytes in such great numbers that the total amount of mitochondrial DNA is about 300 times the amount of chromosomal DNA (Chapter 1, Webb & Smith, 1977), the mitochondria should contribute more to total RNA synthesis in oocytes than they do in somatic cell types. Therefore the possibility exists that a significant proportion of the poly(A)$^+$RNA synthesized in stage 6 oocytes is mitochondrial.

Figure 4-14 presents the results of an experiment which compares the effects of enucleation and microinjection of the drug ethidium bromide (6 μg/ml) on the incorporation of radioactive GTP into total RNA and oligo (dT) bound RNA in stage 6 oocytes. Ethidium bromide is believed to bind to DNA and in so doing extend the helical structure and it is thought that in circular DNAs (eg mitochondrial DNA) this distortion of the helix reduces or prevents transcription (Vesco & Penman, 1969; Craig & Piatigorsky, 1971). Study of Figure 4-14A allows the following observations to be made concerning the effect of ethidium bromide on total RNA synthesis:

(1) In these oocytes healing after microinjection was poor and this may account for the slight deviation from linearity observed in the control oocytes.

(2) Ethidium bromide at 6 μg/ml inhibits the rate of total RNA synthesis only slightly for short times but after longer times the inhibition seems to increase. Nevertheless in the presence of ethidium bromide at this concentration over 50% of the control incorporation of radioactive GTP had occured at 20 hours.

(3) Enucleation reduces the rate of total RNA synthesis dramatically (by 75%) within 5 hours and little or no incorporation occurs after this time. This residual synthesis up to 5 hours seems quite high but may be due in part to the follicle sheaths which were not removed in this experiment, though the follicle cells would be expected to continue incorporating GTP beyond 5 hours.

(4) Ethidium bromide (6 μg/ml) and enucleation together essentially reduced the incorporation of GTP to zero.

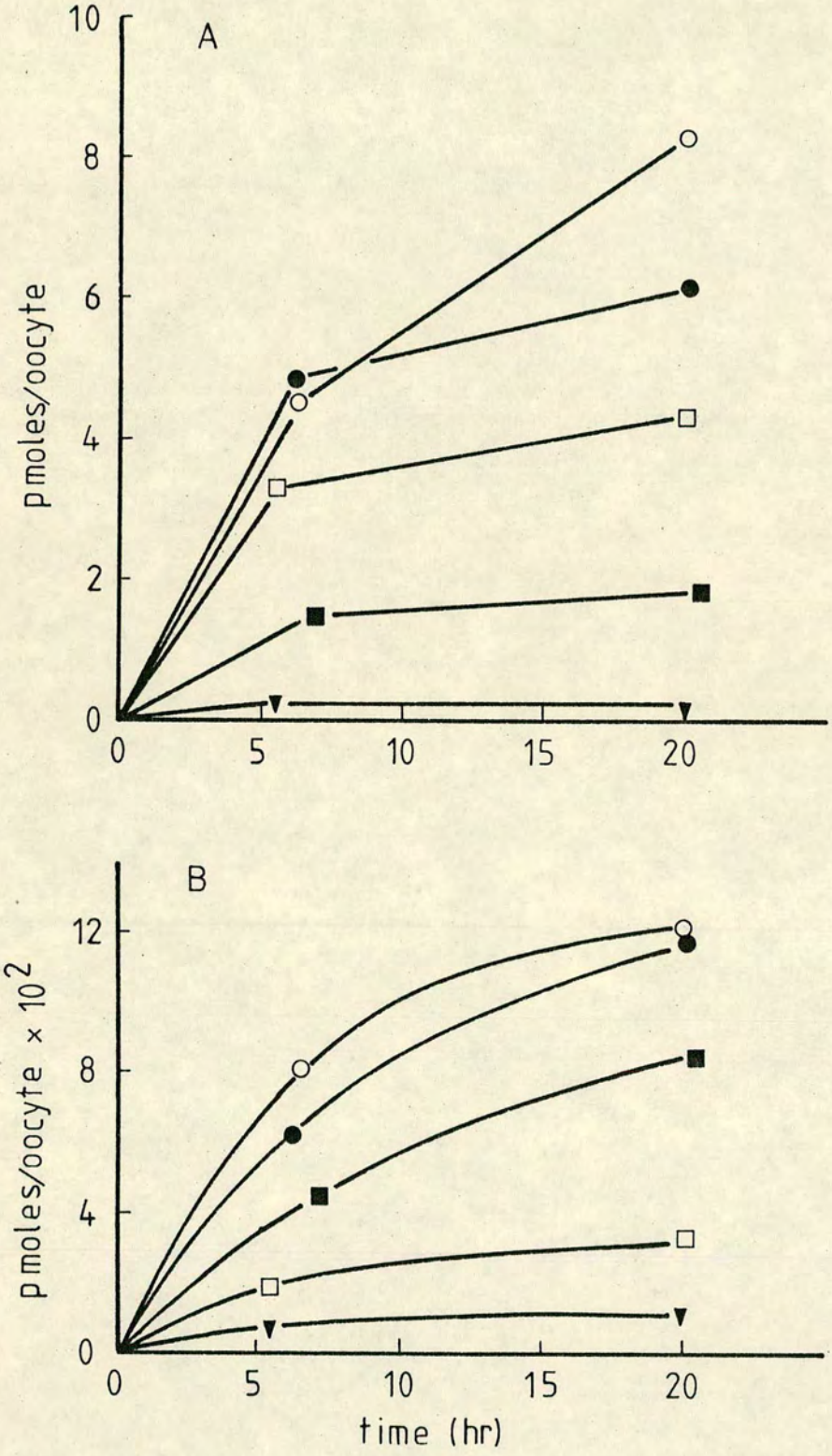

A
B
pmoles/oocyte
pmoles/oocyte x 10²
time (hr)
0
2
4
6
8
10
12
5
15
20

Figure 4-14 Effects of enucleation and ethidium bromide on total RNA and oligo (dT) bound RNA synthesis in stage 6 oocytes

Approximately 600 stage 6 oocytes were stripped from an ovary and about 200 of these were enucleated as described in materials and methods section (f). A total of 100 enucleated and 100 control oocytes were each microinjected with 1 μCi of ^{3}H-GTP in batches of 25 (materials and methods section (d)) and incubated in MBX for 6 hours or 20 hours. A total of 100 enucleated and 100 control oocytes were first injected with 20 nl of an ethidium bromide solution (300 μg/ml) before a second injection of 1 μCi of ^{3}H-GTP as above. The intracellular ethidium bromide concentration was estimated to be 6 μg/ml since the volume of a stage 6 oocyte is about 1,000 nl (Scheer, 1973) and consequently ethidium bromide injected oocytes were incubated in MBX containing ethidium bromide at 6 μg/ml. At either 6 hours or 20 hours oocytes were washed several times in MBX and homogenized in 2 ml of modified Kirby buffer and the RNA extracted as described in section (g) of materials and methods. Samples of incubation medium and homogenate were taken for estimation of the specific activity of the ^{3}H-GTP (Figure 4-2). Samples of total RNA and oligo (dT) bound RNA (prepared from the total RNA as described in materials and methods section (h)) were precipitated with TCA and the radioactivity determined. Data was converted to pmoles/oocyte incorporated using the estimated specific activity of 1,400 cpm/pmole.

(A) Incorporation into total RNA

(B) Incorporation into oligo (dT) bound RNA

(○) control oocytes

(□) ethidium bromide treated

(■) enucleated oocytes

(▼) enucleated and ethidium bromide treated

(●) sum of (□) and (■).

(5) The sum of the curves for the incorporation into total RNA in enucleated oocytes and in ethidium bromide treated oocytes is close to the curve for control oocytes. This suggests that enucleation and ethidium bromide are affecting different types of RNA synthesis.

These results for total RNA synthesis must be contrasted with those in Figure 4-14B for oligo (dT) bound RNA synthesis which show:

(1) The control curve for oligo (dT) bound RNA saturates at around 20 hours and is consistent with earlier data.

(2) Enucleation only reduces the incorporation of radioactive GTP into oligo (dT) bound RNA by a small amount and even after 20 hours the incorporation is still about 70% of the control value.

(3) Ethidium bromide at 6 μg/ml has a greater inhibition on oligo (dT) bound RNA synthesis than does enucleation and after 20 hours there is less than 30% of the control incorporation.

(4) Simultaneous use of ethidium bromide and enucleation almost completely stops incorporation of radioactive GTP. Again the residual incorporation may be into the follicle cells.

(5) The sum of the incorporations occuring in the presence of ethidium bromide and after enucleation closely resembles the amount of incorporation in the control oocytes.

These results show that enucleation and ethidium bromide have opposite effects on the synthesis of total RNA and oligo (dT) bound RNA. However, the presence of the follicle sheaths may complicate any conclusions and therefore a similar enucleation experiment was performed on oocytes which had their follicle sheaths removed after incubation. Figure 4-15 presents the results of this experiment which was entirely consistent with that above. The following conclusions can be drawn from these experiments.

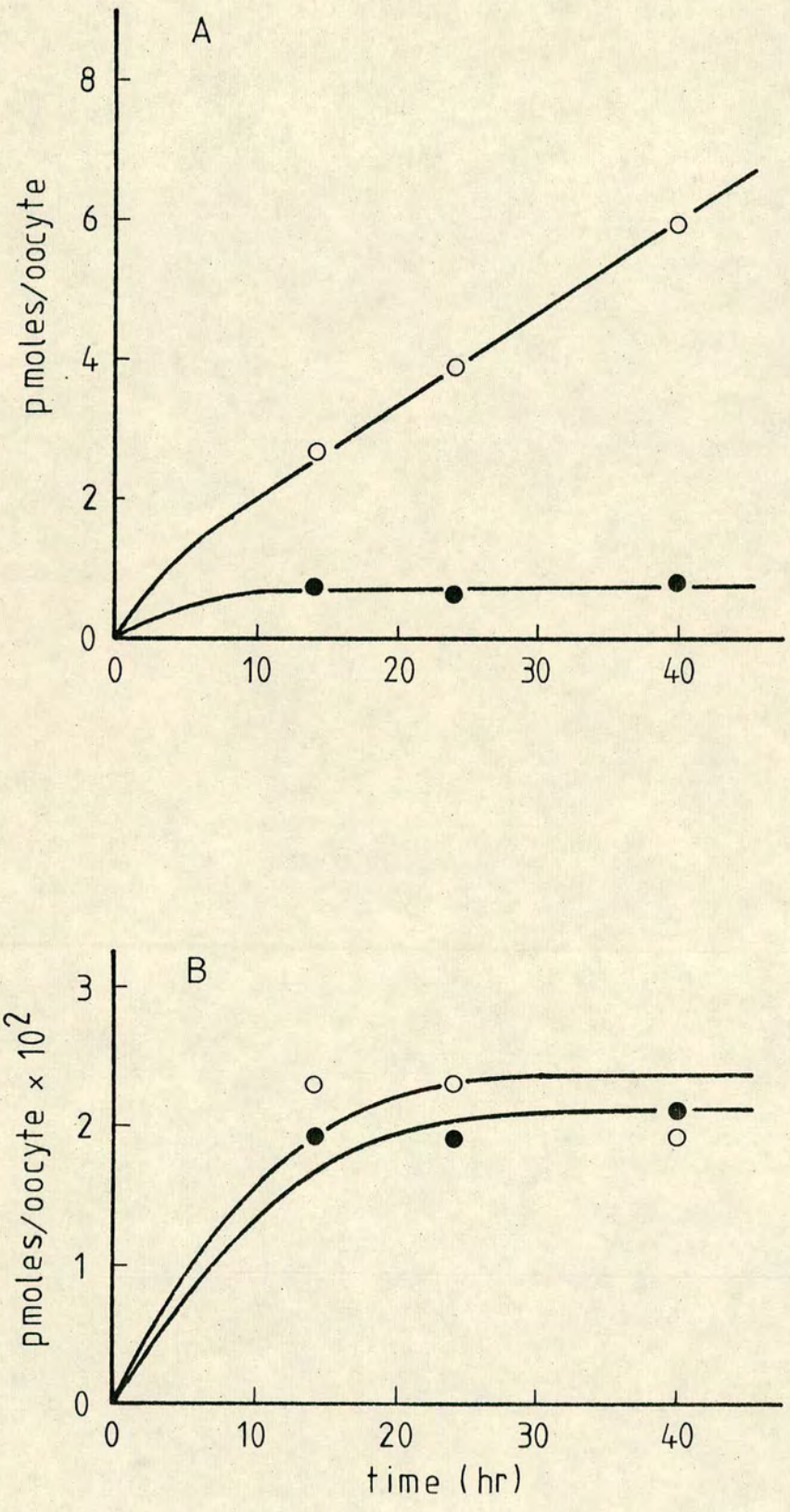

A
B
pmoles/oocyte
pmoles/oocyte x 10²
time (hr)
0
2
4
6
8
10
20
30
40
1
3

Figure 4-15 Effect of enucleation on total RNA and oligo (dT) bound RNA synthesis in defolliculated stage 6 oocytes

Approximately 500 stage 6 oocytes were manually stripped from an ovary and half were enucleated as detailed in materials and methods sections (b) and (f). Both the enucleated oocytes and the control oocytes were microinjected, in batches of 25, with 1 μCi of ^{3}H-GTP each and incubated in 2 ml of MBX for various times (materials and methods section (d)). At the end of the incubations all oocytes were washed several times and the follicle layers were removed and the oocytes homogenized in 2 ml of modified Kirby buffer (materials and methods section (e)). Samples of homogenate and incubation medium were taken to give an estimate of the specific activity of the ^{3}H-GTP (see Figure 4-2), RNA was extracted and oligo (dT) bound RNA was prepared as detailed in materials and methods sections (g) and (h). Samples of total RNA and oligo (dT) bound RNA were precipitated with TCA and the radioactivity present converted to pmoles using the estimated specific activity of 1,500 cpm/pmole.

(A) Incorporation into total RNA

(B) Incorporation into oligo (dT) bound RNA

(O) control oocytes, (●) enucleated oocytes

The presence of follicle sheaths does not substantially affect the results obtained. There is a small amount of incorporation of radioactive GTP into total RNA in enucleated oocytes which ceases within 5 hours of injection. This RNA has been analysed on sucrose gradients and is compared with total RNA from control oocytes in Figure 4-16. From this analysis it appears that 4S/5S, 18S, 28S and heterogeneous RNA species are all labelled in enucleated oocytes for up to 5 hours after enucleation, and the profiles are similar to those of control oocytes. This is unlikely to be due to some oocytes not being enucleated since the method involves removing each individual nucleus manually and furthermore the synthesis of RNA should be maintained and always represent the same proportion of the control oocyte incorporation. One explanation could be that occasionally part of the germinal vesicle, or some of its contents, may remain in the oocyte after enucleation and continue synthesis of RNA. If this is the case then the synthetic activity of this material is lost with time. A further explanation could be that the injected radioactive GTP somehow associates with all RNA species present in the enucleated oocyte. If this is the case then conclusions about the synthesis of RNA in enucleated oocytes must be made with caution.

The incorporation into oligo (dT) bound RNA is reduced by less than 30% by removing the oocyte nucleus. There are two explanations which could account for this observation. Firstly, the majority of the oligo (dT) bound RNA could be synthesized in the cytoplasm, presumably by the mitochondria, and consequently enucleation would have little effect on incorporation into this RNA. Secondly, in the intact oocyte, a small proportion only of the total oocyte oligo (dT) bound RNA might be synthesized by the mitochondria, the rest being nuclear synthesis.

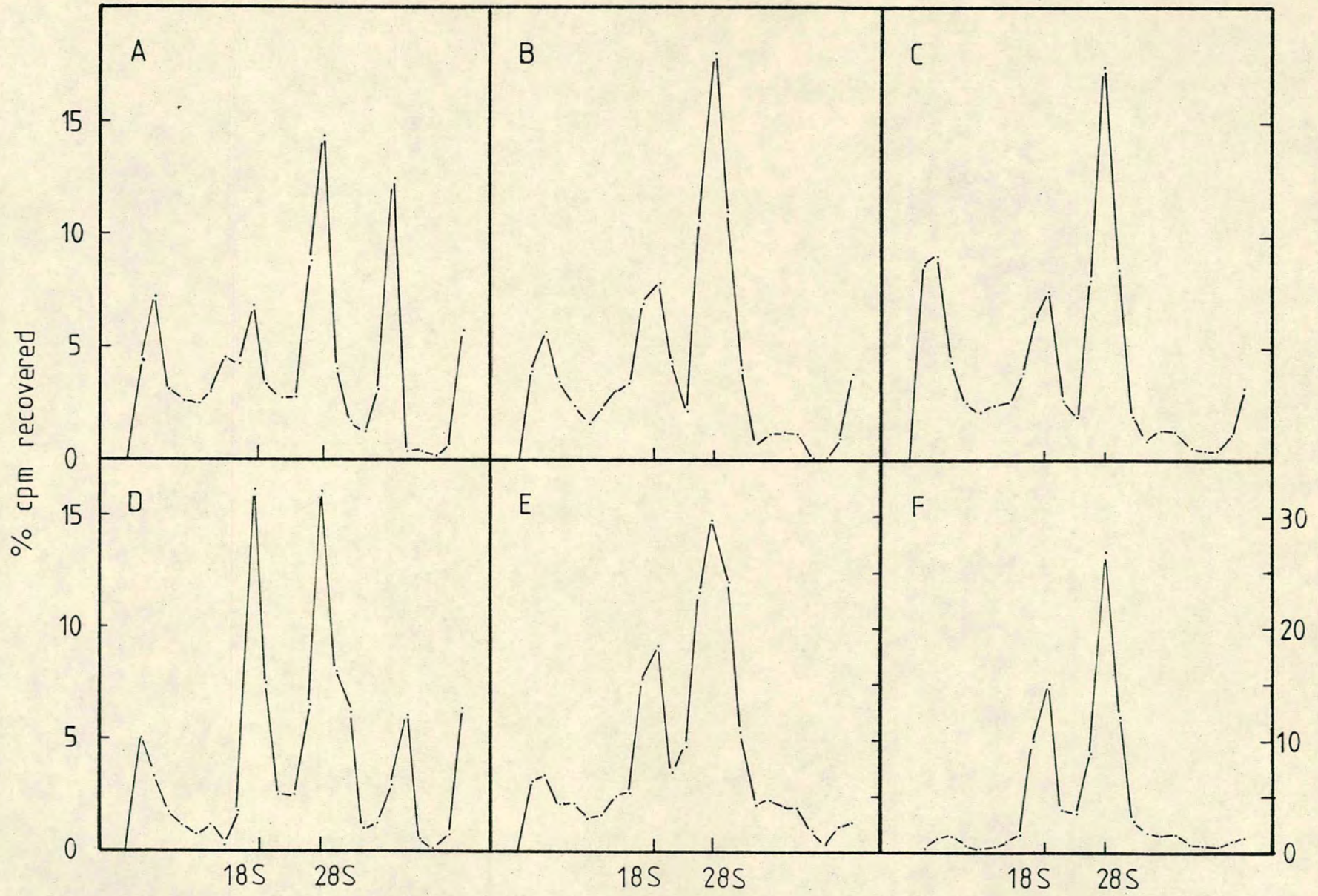

A
B
C
D
E
F
% cpm recovered
0
5
10
15
20
30
18S
28S

Figure 4-16 Sucrose gradient analysis of newly synthesized RNA from enucleated and control stage 6 oocytes

Samples of ^{3}H-GTP labelled total RNA from the experiment described in Figure 4-15 were sedimented in 11.5 ml linear 7-30% sucrose gradients in NETS buffer at 40,000 rpm for 4½ hours at 25°C in an MSE 6 x 14 ml Ti rotor. The gradients were analysed, fractionated and the radioactivity determined as described in materials and methods sections (i) and (m). Centrifugation was from left to right and the last fraction is the pellet. Recovery of loaded cpm was over 65% and the figures show the percentage per fraction of the total recovered cpm.

Enucleated oocytes

(A) 14 hours incubation, 1,800 cpm recovered

(B) 24 hours incubation, 1,100 cpm recovered

(C) 40 hours incubation, 1,600 cpm recovered

Control oocytes

(D) 14 hours incubation, 2,500 cpm recovered

(E) 24 hours incubation, 3,600 cpm recovered

(F) 40 hours incubation, 4,550 cpm recovered.

However, if enucleation resulted in an activation of mitochondrial oligo (dT) bound RNA synthesis, it would be possible to obtain this result. Since it would be hard to explain why enucleation could produce a hundred fold activation of mitochondrial oligo (dT) bound RNA synthesis, the proportion of total oocyte oligo (dT) bound RNA synthesis that might be mitochondrial, would have to be nearer 10-50% than 1%.

If this second hypothesis is correct it might be possible to detect a difference in sedimentation properties between enucleated and control oocyte newly synthesized oligo (dT) bound RNA, since the mitochondrial genome should only code for a few mRNAs. Figure 4-17 presents the results of such an experiment. It is clear that no difference in sedimentation properties is observed.

Another way of trying to detect a difference in sedimentation between mitochondrial and total oocyte oligo (dT) bound RNA is to compare the poly (U) binding activity in gradients of total RNA prepared from mitochondrial fractions and other oocyte fractions. Mitochondria were banded on a discontinuous sucrose gradient as described in materials and methods section (i) and RNA was extracted from this band as well as from the rest of the gradient. These RNA samples were then run on 7-30% sucrose gradients and the fractions were assayed for poly(A) by hybridization to ^{3}H-poly(U). Figure 4-18 presents the results of this experiment and also gives the percentage poly (U) binding activity and the percentage SDH activity in the oocyte fractions. These mitochondrial fractions contained about $\frac{1}{4}$ of the total oocyte SDH activity and 5% of the total oocyte poly (U) binding activity. There is a distinct difference between these two fractions. The poly (U) binding activity in the mitochondrial fraction being much more

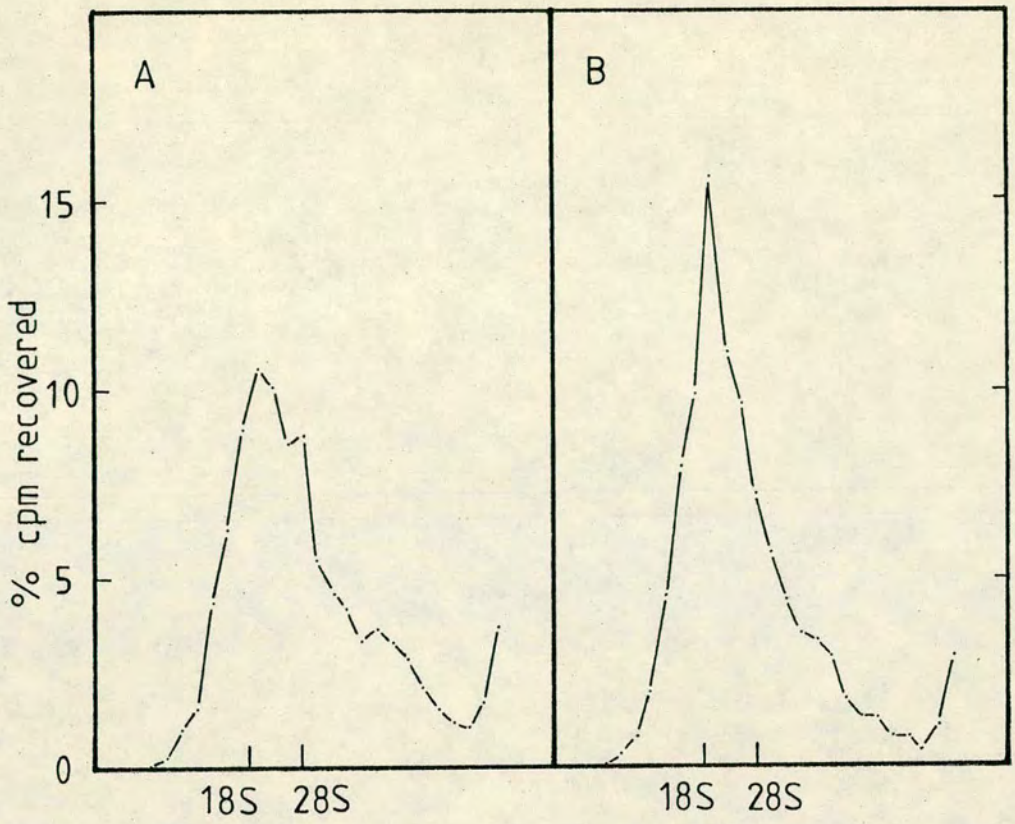


Figure 4-17 Sedimentation analysis of newly synthesized oligo (dT) bound RNA from enucleated and control stage 6 oocytes

Samples of oligo (dT) bound RNA from the experiment described in Figure 4-15 were sedimented, analysed and the data plotted exactly as in Figure 4-16. Recovery of loaded cpm was over 85%

(A) control oocytes incubated for 40 hours, 2,100 cpm recovered.

(B) enucleated oocytes, pooled 14, 24 and 40 hour samples, 2,200 cpm recovered.

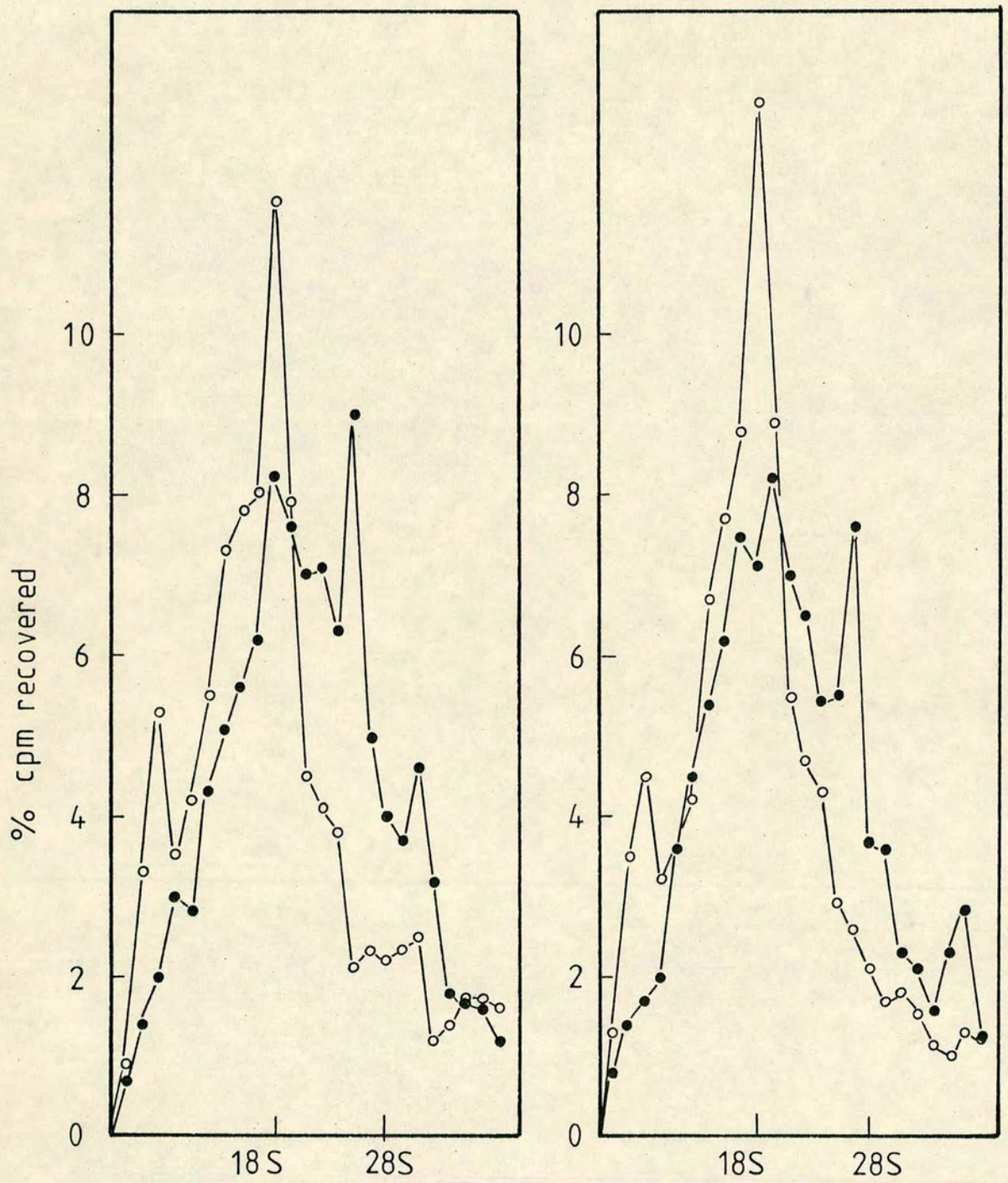

% cpm recovered
0
2
4
6
8
10
18S
28S
0
2
4
6
8
10
18S
28S

Figure 4-18 Sedimentation analysis of poly(A)$^+$RNA from a mitochondrial fraction measured by poly(U) hybridization

Using the method outlined in section (i) of materials and methods, 250 oocytes were homogenized, the sample split in two and mitochondria banded on discontinuous sucrose gradients. The SDH content of samples of total homogenate, mitochondrial fractions and the remainder of the gradients were measured as detailed in materials and methods section (n) and total RNA was extracted (section (g) of materials and methods). Samples of total RNA were sedimented in 11.5 ml linear 7-30% sucrose gradients using conditions exactly as described in Figure 4-17. The poly(U) binding activity of samples of total RNA and of gradient fractions was determined as described in materials and methods section (k). In the figures data is expressed as the percentage per fraction of the total cpm of poly(U) recovered and the last fraction is the pellet. (A) and (B) are duplicates and over 90% of the loaded cpm were recovered.

(o) mitochondrial fraction

(•) remainder of gradient fraction.

Sample	SDH Assay		Poly (U) Assay	
	Change in OD	% Total change in OD	cpm recovered	% Total cpm recovered
"remainder" fraction A	7.62	77%	115,080	95%
mitochondrial fraction A	2.30	23%	6,739	5%
"remainder" fraction B	8.94	76%	181,120	94%
mitochondrial fraction B	2.80	24%	11,966	6%

The total change in OD in fractions A and B is 21.66 which was 88% of that of 24.64 measured for the total homogenate.

homogeneous, sedimenting at about 18S or smaller. The poly (U) binding activity of the other fraction is more heterogeneous and it contains more rapidly sedimenting poly(A) containing molecules. The total RNA in the mitochondrial fractions showed no signs of degradation and the reproducable patterns of poly (U) binding down these mitochondrial gradients argues that this size difference is not due to degradation of the mitochondrial fractions.

The sedimentation profiles of newly labelled oligo (dT) bound RNA in Figure 4-17 for enulceated and control oocytes and in Figure 4-9 for cytoplasm resemble more closely the poly (U) binding profile of the mitochondrial fraction than that of the rest of the oocyte (Figure 4-18), and since it has been shown earlier that the half-life of newly labelled oligo (dT) bound RNA is about 5-10 hours, it is not unreasonable to expect this newly labelled RNA to have the steady state size by 20 hours (the time points in Figures 4-17 and 4-9).

In Figure 4-18 about 5% of the total oocyte poly (U) binding activity was isolated in this mitochondrial fraction. A gradient profile of the total RNA in this fraction showed that about 2% of the total oocyte 18S and 28S rRNA contaminated the mitochondrial RNA. The degree of contamination of the mitochondrial poly (U) binding activity by poly (U) binding activity from the rest of the oocyte is likely to be of the same order which would mean that a significant proportion of the poly (U) binding activity in the mitochondrial fraction was not of mitochondrial origin. This problem of RNA cross-contamination also observed by other workers (Webb et al., 1975) meant that no firm conclusions could be drawn from experiments in which newly labelled oligo (dT) bound RNA was isolated from oocyte mitochondrial fractions.

(e) Discussion

Using the microinjection technique it was possible to obtain an effectively constant intracellular specific activity of injected radioactive ATP or GTP. It was thus possible to show that stage 6 oocytes synthesize and accumulate stable RNA linearly for at least 110 hours after microinjection. Most of this RNA was 18S and 28S rRNA and it was accumulated in the oocyte cytoplasm.

The initial rate of incorporation of labelled NTP was at least $2\frac{1}{2}$ times greater than the incorporation into stable RNA and it was concluded that a minimum of 70% of the newly labelled RNA is unstable and turns over with an average half-life of less than 4 hours. Since there was a lag before the appearance of label in the cytoplasm this rapidly turning over RNA must be located in the nucleus. At all times, analysis of the newly labelled nuclear RNA showed that heterogeneous RNA accounted for at least 50% with rRNA precursors accounting for most of the rest. The data presented here therefore agrees with the conclusions of Anderson & Smith (1977) that stage 6 oocytes of Xenopus laevis synthesize large amounts of heterogeneous nuclear RNA which has a short half-life. These authors suggested that about 5% of this hnRNA was relatively stable (half-life > 90 hours) and accumulated in the cytoplasm. This would mean that 15% of the labelled cytoplasmic RNA would be heterogeneous. The data presented here showed that there was some apparently stable heterogeneous RNA in the cytoplasm and it accounted for no more than 20% of the total cytoplasmic labelled RNA.

In this investigation emphasis was placed on studying the synthesis of poly(A)$^+$RNA which is generally accepted to be mRNA. It was shown that incorporation of radioactive NTP into oligo (dT)

bound RNA levelled off in about 20 hours and no stable component was observed. The steady state amount accumulated corresponded to 0.2-1.0% of the 40 ng of stored poly(A)$^+$RNA and both the newly synthesized and stored poly(A)$^+$RNA had similar sedimentation properties (Rosbash & Ford, 1974).

The cellular site of synthesis of the labelled poly(A)$^+$RNA detected in this investigation was probed in two ways. The method yielding the more conclusive data was that of enucleation. Relative to control oocytes, after 20 hours or more of incubation enucleated oocytes had synthesized less than 20% of the total RNA but over 70% of the poly(A)$^+$RNA. The sedimentation properties of control and enucleated oocyte labelled poly(A)$^+$RNA were indistinguishable and also similar to the poly (U) binding activity profile of RNA prepared from an oocyte mitochondrial fraction. This data argues quite strongly that the newly synthesized stage 6 oocyte poly(A)$^+$RNA is mainly mitochondrial in origin. In this connection, Webb et al. (1975) have reported that Xenopus laevis oocyte mitochondria synthesize heterogeneous RNA some 30% of which may be polyadenylated.

The second method used to investigate the cellular distribution of newly labelled poly(A)$^+$RNA was that of germinal vesicle isolation in a modified isolation medium. This procedure yielded the following information about oligo (dT) bound RNA synthesis:

(1) Using radioactive GTP as label over 85% of the incorporation into oligo (dT) bound RNA was into the cytoplasmic fraction at all times.

(2) Using radioactive ATP 40%, or more at early times, of the total incorporation was into germinal vesicle oligo (dT) bound RNA.

(3) Sucrose gradient analysis of this RNA showed that cytoplasmic oligo (dT) bound RNA always closely resembled that of the intact

oocyte but that germinal vesicle ATP labelled oligo (dT) bound RNA sedimented reproducably at about 7S. Total nuclear RNA was not degraded under these conditions.

(4) This newly labelled 7S germinal vesicle material was shown to be largely poly(A) with a heterogeneous size on 10% SDS/acrylamide gels (30-150 A residues).

(5) The poly(A) of cytoplasmic oligo (dT) bound RNA ran more homogeneously on these gels with a large peak at about 60 A residues.

(6) This 7S germinal vesicle poly(A) was not detected in the intact oocyte despite the large proportion of the total incorporation that it represented.

(7) There is no evidence to suggest that there is more incorporation into oligo (dT) bound RNA in intact oocytes than in oocytes fractionated in this way (Table 4-3).

(8) About 10% of the oocyte poly (U) binding activity is in the germinal vesicle, and the newly labelled germinal vesicle poly(A) would represent about 5% of this if it is labelled throughout its entire length.

The observations presented above are difficult to reconcile and any explanation must distinguish between the following alternatives:

(1) That this 7S germinal vesicle poly(A) exists as such in intact oocytes.

(2) That this 7S material is produced by degradation of larger molecules during the isolation procedure.

(3) That it is produced as 7S poly(A) during isolation.

Alternative (1) is consistent with the observation that GTP does not label nuclear oligo (dT) bound RNA, providing the newly synthesized

oligo (dT) bound RNA is mostly mitochondrial. Nuclear newly labelled total RNA is not degraded during the isolation procedure and this argues that any newly synthesized oligo (dT) bound RNA should not be degraded. Therefore if this 7S germinal vesicle poly(A) is present in intact oocytes, why is it not possible to observe it in the intact oocyte when there is roughly equal incorporation of ATP into both germinal vesicle and cytoplasmic oligo (dT) bound RNA fractions?

Alternative (2) gets round this problem by suggesting that the 7S germinal vesicle poly(A) is not present as such in intact oocytes but is produced from a larger species of RNA during nuclear isolation. This hypothesis requires that the germinal vesicle contains more poly(A)$^+$RNA than the cytoplasm but that on isolation only the poly(A) tail is recovered. If the size of this germinal vesicle poly(A)$^+$RNA is similar to that in the cytoplasm then to obtain as much incorporation into nuclear poly(A) as into cytoplasmic poly(A)$^+$RNA would require 5-15 times more poly(A)$^+$RNA in the nucleus than in the cytoplasm.

Table 4-3 shows that in a number of experiments the steady state amount of oligo (dT) bound RNA expressed as a fraction of the rate of stable RNA synthesis for intact oocytes and for fractionated oocytes are similar and do not support this hypothesis. Furthermore, in steady state conditions ATP incorporation into poly(A) in the germinal vesicle accounts for about 7% of the total nuclear incorporation. This hypothesis would require that about 35% of the nuclear incorporation was into poly(A)$^+$RNA. In a cell which appears not to be accumulating poly(A)$^+$RNA, but which is capable of its long term storage and which is to some extent specialized for the production of rRNA this value would seem too high. The fact that the total RNA, both newly labelled

and unlabelled, isolated from these germinal vesicles is not apparently degraded and 32S rRNA precursor molecules are observed, suggests that if this hypothesis is correct then nuclear poly(A)$^+$RNA is very specifically degraded. Perhaps it might lack certain protein molecules which aid its stabilization? Whatever the cause conditions would have to exist which permit the complete degradation of presumably a whole population of poly(A)$^+$RNA molecules leaving only the poly(A) tails but which do not permit degradation of a number of other RNA species some of which are quite sensitive to degradation (32S rRNA precursor).

Hypothesis (3) requires that the production of the 7S germinal vesicle poly(A) in these experiments occurs in some way as a result of the cellular disruption caused during the process of germinal vesicle isolation. Chris Darnbrough (personal communication) has shown that stage 6 oocytes of Xenopus laevis possess the capacity to increase their content of poly (U) binding activity by 50% during the 6 hours after exposure to progesterone before germinal vesicle breakdown (i.e. in vitro maturation). This may be due to the synthesis of new poly(A)$^+$RNA molecules and/or to extension of the poly(A) tails of pre-existing poly(A)$^+$RNA molecules. The important point is that the stage 6 oocyte has this capacity and it is possible that during germinal vesicle isolation nuclear polyadenylases may be activated to produce either new poly(A) molecules or to extend pre-existing poly(A) molecules. Indeed, if the germinal vesicle contains 10% of the total oocyte poly(U) binding activity and poly(A) molecules are approximately 100 A residues long, then addition of 10 A residues to all these molecules could account for the observed incorporation of labelled ATP into the germinal vesicle. This process could account for the heterogeneous profile of the newly labelled germinal vesicle poly(A)

molecules if there was sufficient heterogeneity in their sizes prior to extension. As mentioned above the amount made would represent roughly 5% of the germinal vesicle poly (U) binding activity or about 0.5% of the total oocyte poly (U) binding activity. If the increase in poly (U) binding activity observed by Chris Darnbrough occured linearly throughout the 6 hours then 0.5% of the total would be made in $3\frac{1}{2}$ minutes. It would therefore be possible for the germinal vesicle to make this new poly(A) during the germinal vesicle isolation procedure since isolated nuclei are not placed in Kirby's medium until 30-90 seconds after the cytoplasmic membrane is pierced.

A different mechanism for this hypothesis could be that the stage 6 oocyte germinal vesicle contains large quantities of polyadenylases which are inactive in the intact oocyte. Removal of the germinal vesicle from its cytoplasmic environment might cause activation of these enzymes and poly(A) or poly(A)-rich RNA of about 7S might result. If this is the case, the stage 6 oocyte cytoplasm might contain an inhibitor of nuclear polyadenylases.

Hypothesis (3) would require that the sum of the nuclear and cytoplasmic incorporation of labelled ATP into poly(A)$^+$RNA should be greater than that of the intact oocyte by $1\frac{1}{2}$-2 times. Using the same female, this experiment has been done for one time point only and the result shows little difference if any. Another minor objection to this hypothesis is that the newly synthesized poly(A) size profile for the intact stage 6 oocyte does not closely resemble the relatively homogeneous cytoplasmic profile of Figure 4-12. It in fact resembles more closely the sum of the nuclear and cytoplasmic poly(A) size profiles. The data for the intact oocytes was obtained by Chris Darnbrough using a different female and this may partly account for the difference.

It is obvious that none of these hypotheses explains satisfactorily all the observations. There are more objections to hypothesis (2) however which therefore makes it the least likely explanation. It must be concluded then, that either there is free poly(A) turning over in the germinal vesicle of stage 6 oocytes or that results obtained by isolation of the germinal vesicle must be interpreted with caution since it may be that the germinal vesicle isolation procedure itself can trigger off unusual poly(A) metabolism.

Since a primary objective of this thesis was to compare RNA synthesis in various stages of oogenesis it was resolved not to spend further time trying to clarify this problem concerning nuclear poly (A). In the following Chapter attempts to investigate RNA synthesis during early oogenesis are presented.

CHAPTER 5

RNA Synthesis in Previtellogenic Oocytes

(a) Introduction

The observation that the amount of poly(A)$^+$RNA present in oocytes of Xenopus laevis remains relatively constant from early vitellogenesis to ovulation (Rosbash & Ford, 1974) suggests that the synthesis of these molecules occurs during previtellogenesis. Ford et al. (1977) have shown that in Xenopus laevis oocytes, poly(A)$^+$RNA, which is very stable (half-life 2-4 years), is made in vivo at some time during early oogenesis.

In Chapter 4 it was shown that although some poly(A)$^+$RNA is made in stage 6 Xenopus laevis oocytes incubated in vitro no detectable net storage of poly(A)$^+$RNA molecules occurred and further this RNA may be a different class of poly(A)$^+$RNA to that stored. The observations of Chapter 4 are therefore consistent with the above reports.

It was reasoned that the synthesis of this stored poly(A)$^+$RNA must be occurring in previtellogenic oocytes of stage 1 or early stage 2 and therefore attempts to show the in vitro synthesis of poly(A)$^+$RNA by stage 1 Xenopus laevis oocytes were made. If synthesis of poly(A)$^+$RNA was detected in previtellogenic ovary it might be possible to show differences between the characteristics of newly synthesized previtellogenic oocyte poly(A)$^+$RNA and newly synthesized stage 6 oocyte poly(A)$^+$RNA, and therefore support the conclusion of Chapter 4 that the poly(A)$^+$RNA made in vitro in stage 6 oocytes is not of nuclear origin. The results of these attempts are reported in this Chapter.

(b) RNA synthesis in previtellogenic ovary

Initial experiments investigating RNA synthesis during early oogenesis involved incubating total ovary in MBX containing radioactive adenosine. A number of ovaries had to be used in each experiment and these varied somewhat in their stage of development. However, even ovaries from immature females which contain a few white oocytes (stage 2) are mainly composed of clear oocytes (stage 1) (Ford et al., 1977). It is therefore considered that all the ovaries chosen for these experiments were previtellogenic (stage 1).

Total RNA

Figures 5-1A, B and C present the kinetics of incorporation of radioactive nucleoside into total RNA in previtellogenic ovary. The incorporation is expressed as cpm/ μg of RNA isolated. In all the experiments performed the incorporation of nucleoside into total RNA by whole previtellogenic ovary is linear up to 80 hours of incubation (the longest time point). For linear incorporation into RNA to occur the intracellular precursor specific activity would need to be constant from an early time. Figure 5-2 gives measurements of the total cpm in the ovary homogenate at each of the time points in Figure 5-1A. The data shows that within 6 hours (the earliest time point) the majority of the soluble intracellular cpm have already been taken up from the medium. It is therefore reasonable to assume that the uptake of radioactive nucleoside by previtellogenic ovary is very rapid and that a constant specific activity of RNA precursor is attained very early in the incubation (less than 6 hours).

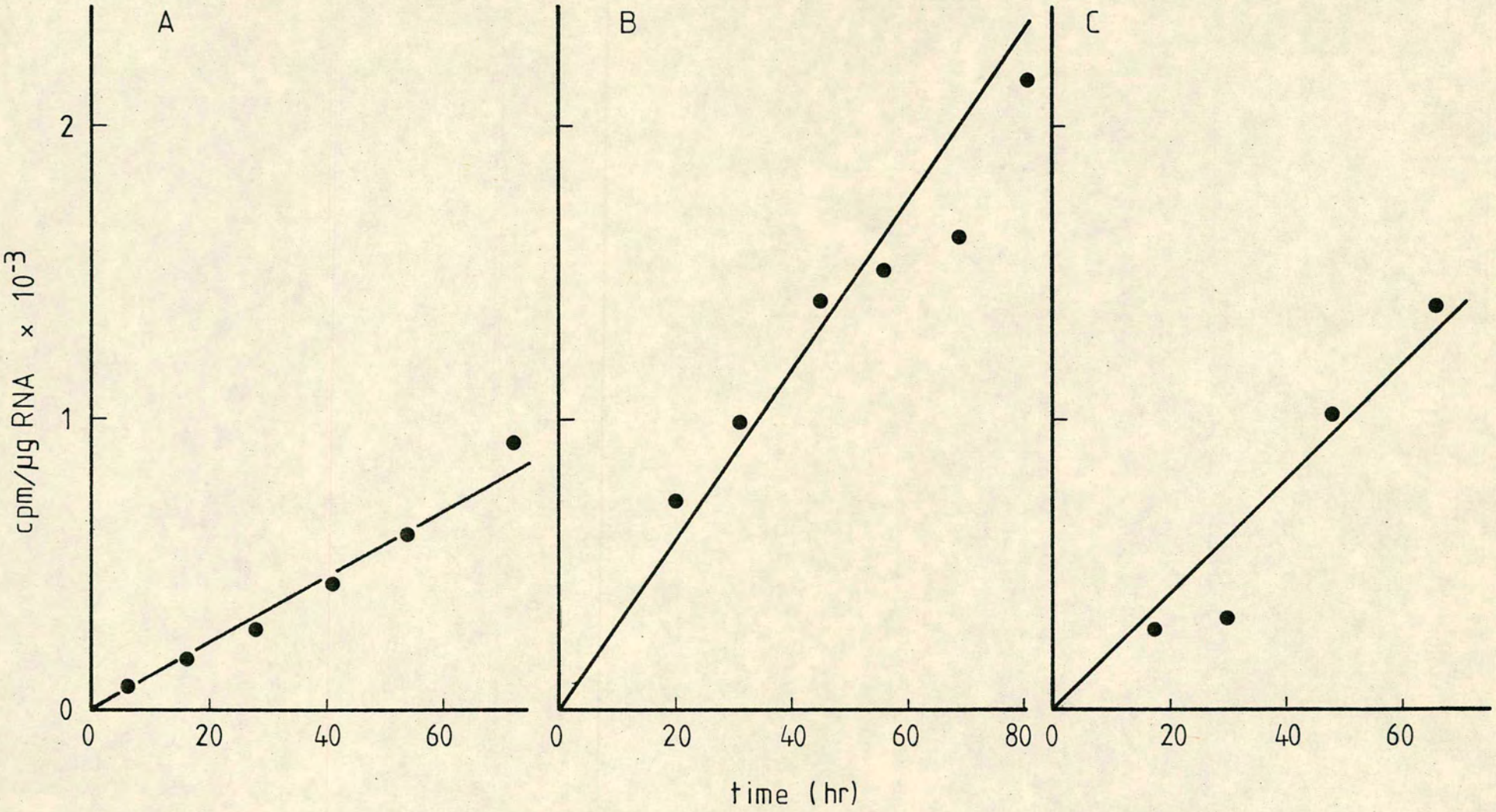

A
B
C
cpm/µg RNA × 10⁻³
0
1
2
0
20
40
60
80
time (hr)

Figure 5-1 Incorporation kinetics for total RNA synthesis *in vitro* by previtellogenic ovary

(A) ^{3}H-adenosine incorporation kinetics

5 immature ovaries (stage 1) were incubated as described in materials and methods section (c) in MBX containing 2-^{3}H-adenosine at 312 μCi/ml. At the times indicated approximately 1/6 of the ovarian material was removed and the RNA extracted (section (g) of materials and methods). The optical densities of samples of total RNA were measured and aliquots of total RNA were precipitated with TCA, the radioactivity being determined as described in materials and methods section (m). The results are expressed as cpm incorporated per μg of total RNA isolated in each sample.

(B) ^{3}H-adenosine incorporation kinetics

This experiment was performed exactly as described in Figure 5-1A except that 4 ovaries were used and the radioactive concentration was 350 μCi/ml. The results are expressed as cpm incorporated per μg of total RNA isolated.

(C) ^{3}H-uridine incorporation kinetics

This experiment was performed exactly as described in Figure 5-1A except that 3 ovaries were incubated in MBX containing 5-^{3}H-uridine at 350 μCi/ml. At various times approximately $\frac{1}{4}$ of the ovarian material was removed and processed as above. The results are expressed in the same way.

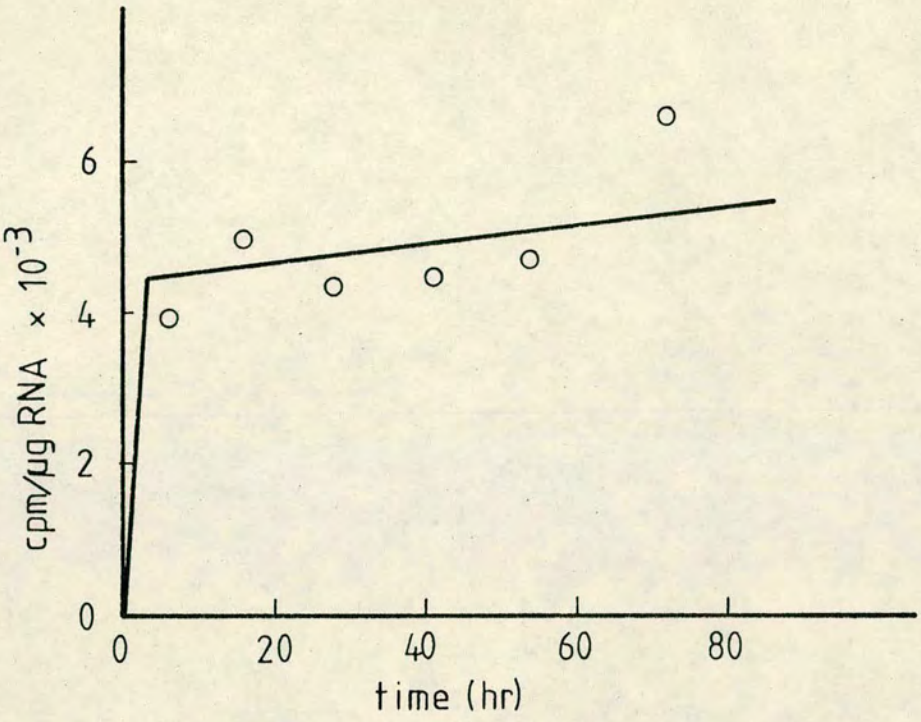


Figure 5-2 ^{3}H cpm in previtellogenic ovary homogenate

In the experiment described in Figure 5-1A aliquots of the previtellogenic ovary homogenate in Kirby buffer were spotted directly onto GF/C filters, dried and the radioactivity determined as described in materials and methods section (m). Correction was made for the quenching effect of Kirby buffer. The results are expressed as cpm present per µg of total RNA extracted.

The RNA synthesized during these experiments by previtellogenic ovary was analysed on sucrose gradients and the results are presented in Figure 5-3. These sets of gradients show that 4S/5S, 18S, 28S and heterogeneous RNA are all synthesized by previtellogenic ovary. Using the method of Girard et al. (1965) the proportions of the incorporation in each species present remains quite constant in each set of gradients suggesting that all these species are mainly stable RNA. The actual proportions of label in each newly synthesized species of RNA in the different experiments varies somewhat but is roughly: 50-60% 4S/5S RNA, 20-25% 18S and 28S RNA, 20-25% heterogeneous RNA. In these gradients the optical density traces of the total ovary RNA showed very little 18S and 28S RNA present, most of the absorption being in the 4S/5S region of the gradient. These results are consistent with earlier work (Ford, 1972; Thomas, 1974; Rosbash & Ford, 1974).

Oligo (dT) bound RNA

In Figure 5-4 the kinetics of incorporation of labelled nucleoside into oligo (dT) bound RNA in previtellogenic ovary is given. In all three experiments the incorporation of nucleoside saturates at about 30 hours. If (as suggested earlier) the specific activity of the precursor is constant then this profile means that most of the oligo (dT) bound RNA made by previtellogenic ovary is unstable with an average half-life of 10-15 hours. The scatter in the results would prevent detection of a small stable component if it were present. Until saturation, the oligo (dT) bound RNA represents about 5% of the incorporation into total RNA in previtellogenic ovary.

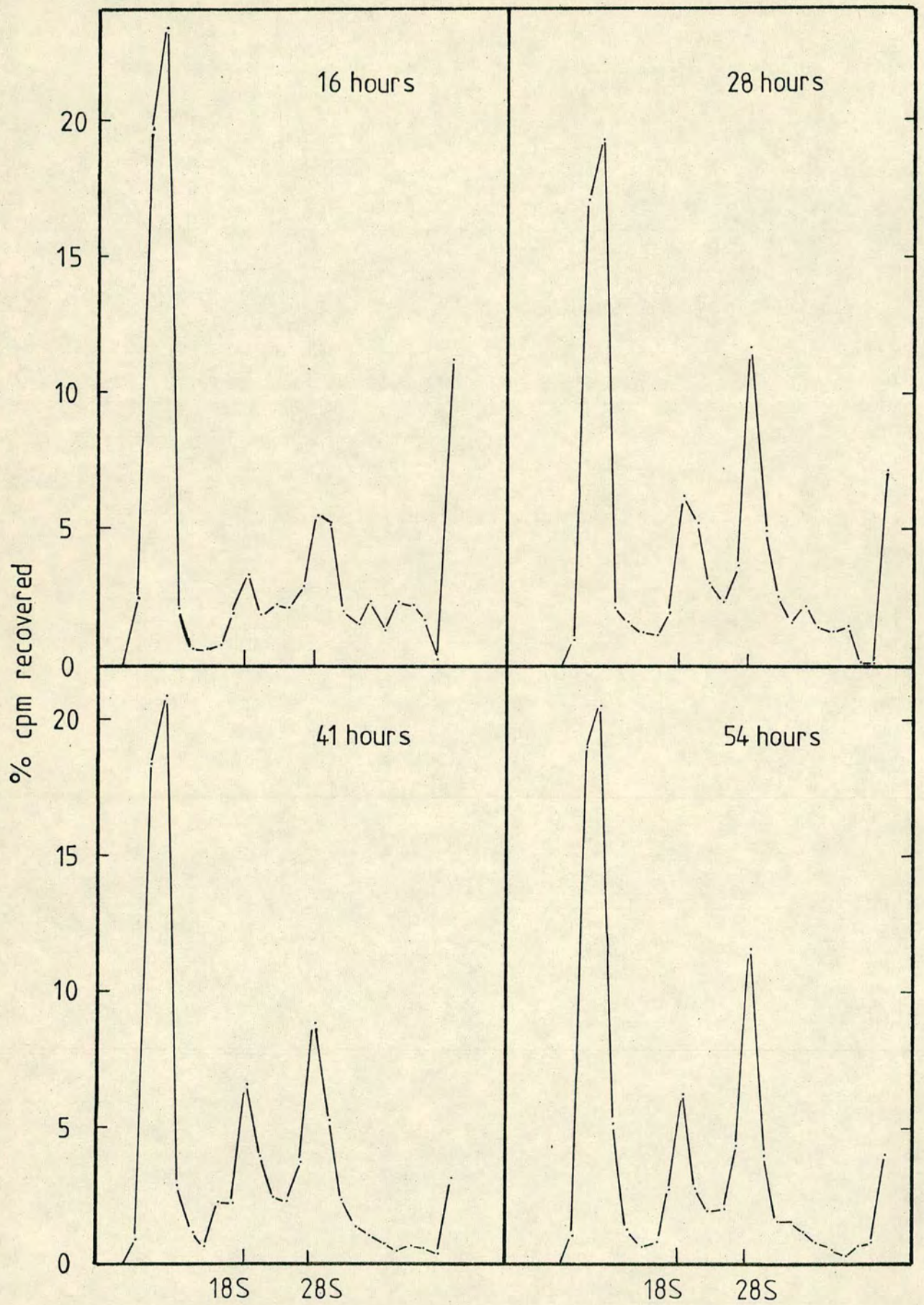

16 hours
28 hours
41 hours
54 hours
% cpm recovered
0
5
10
15
20
18S
28S
18S
28S

Figure 5-3A Sucrose gradient analysis of ^{3}H-adenosine labelled total RNA from previtellogenic ovary

Samples of total RNA from the experiment described in Figure 5-1A were sedimented in 11.5 ml linear 7-30% sucrose gradients in NETS buffer at 40,000 rpm for 4½ hours in an MSE 6 x 14 ml Ti rotor at 25°C. The gradients were analysed and the TCA precipitable radioactivity determined as described in materials and methods sections (i) and (m). Recovery of loaded cpm was 70-95% and the figures show the percentage of the total recovered cpm which is present in each fraction. Centrifugation was from left to right and the last fraction is the pellet. The following cpm were recovered:

16 hours	11,700 cpm;	28 hours	11,700 cpm
41 hours	13,000 cpm;	54 hours	21,700 cpm

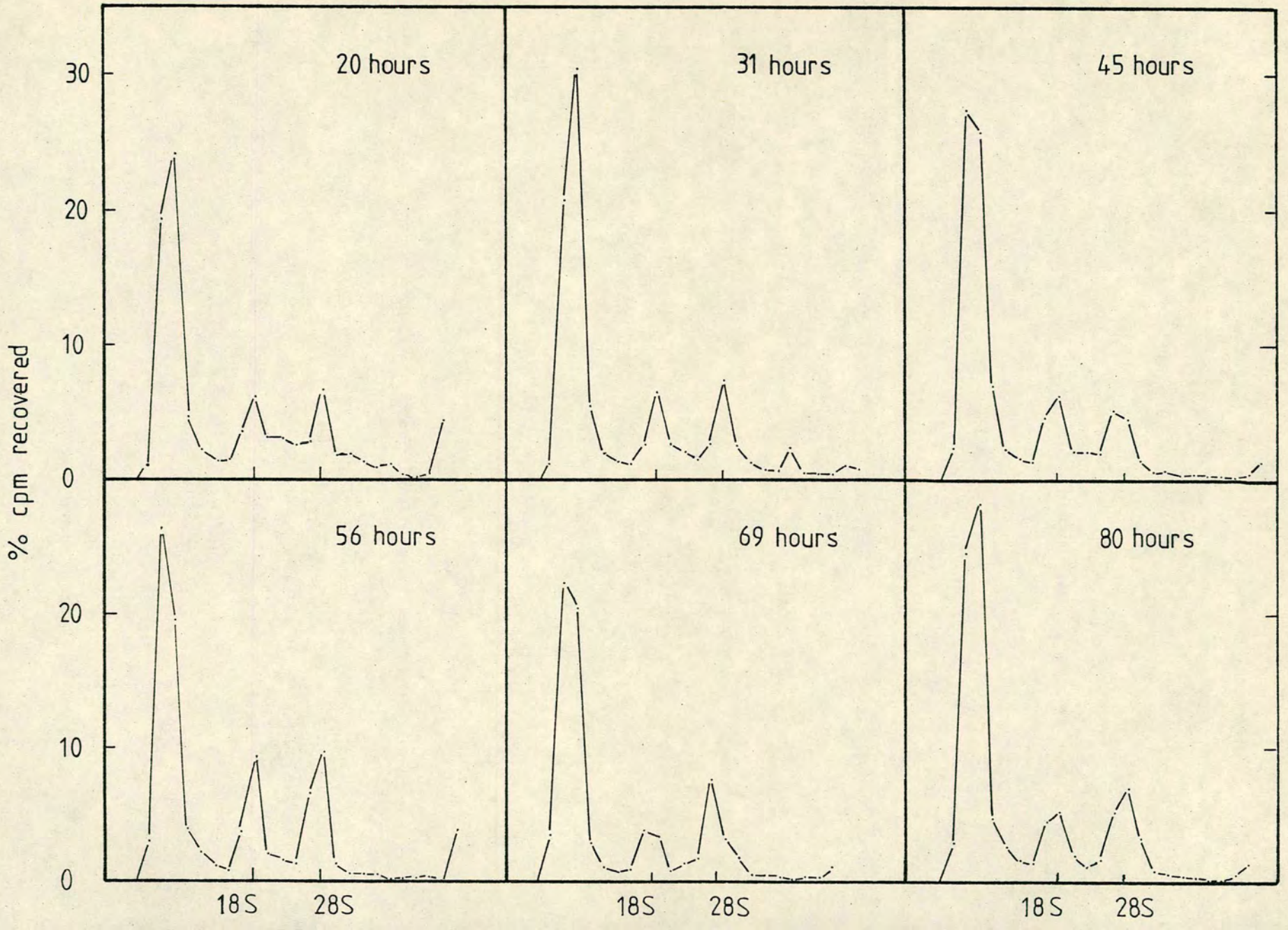

20 hours
31 hours
45 hours
56 hours
69 hours
80 hours
% cpm recovered
30
20
10
0
18S
28S

Figure 5-3B Sucrose gradient analysis of ^{3}H-adenosine labelled total RNA from previtellogenic ovary

This sedimentation analysis was performed exactly as described in Figure 5-3A using samples of total RNA from the experiment described in Figure 5-1B. Recovery of loaded cpm from the gradients was over 70% and the results are presented as in Figure 5-3A. The following cpm were recovered:

20 hours	2,900 cpm;	31 hours	5,900 cpm;	45 hours	4,100 cpm;
56 hours	4,300 cpm;	69 hours	6,200 cpm;	80 hours	5,500 cpm.

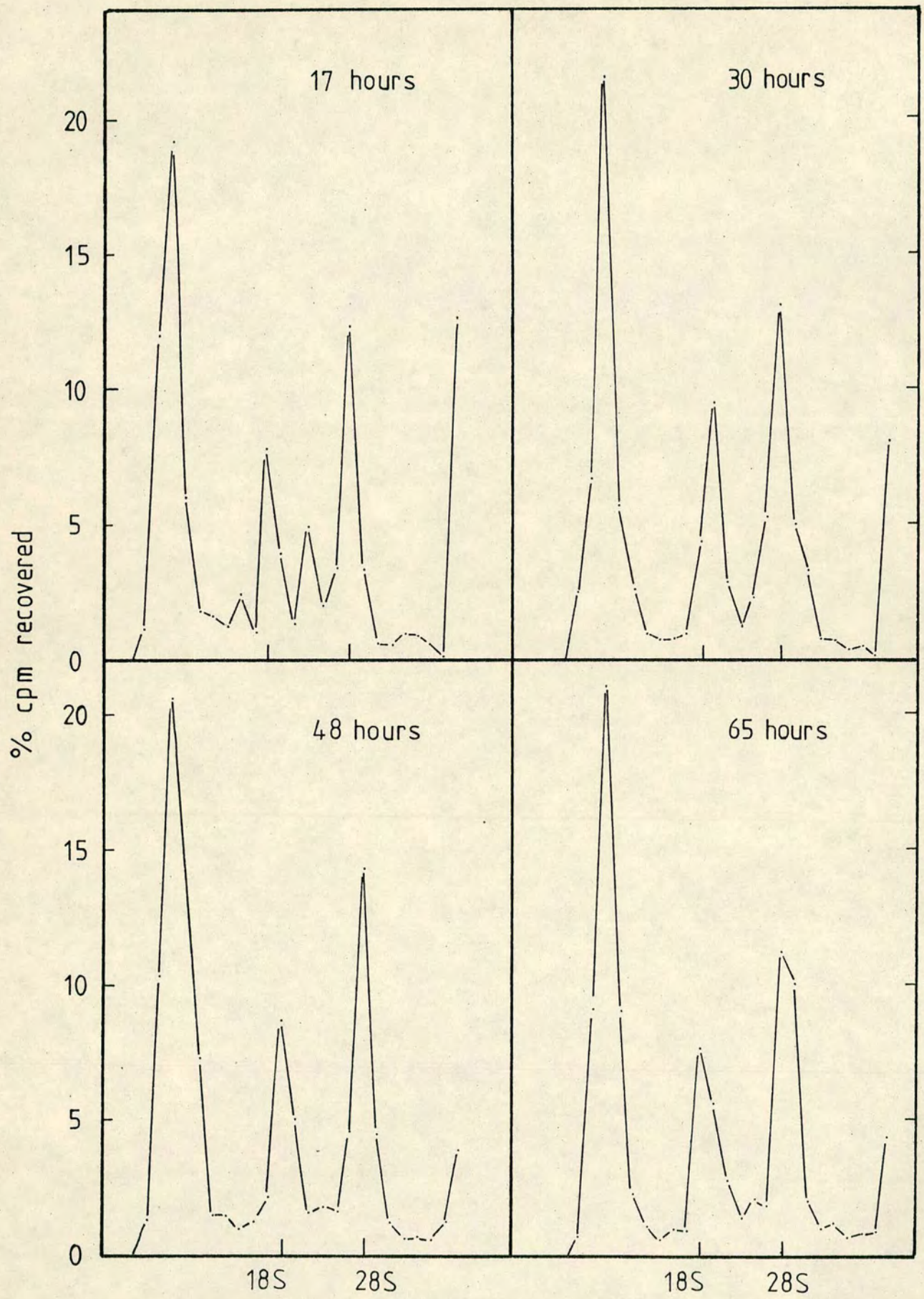

17 hours
30 hours
48 hours
65 hours
% cpm recovered
0
5
10
15
20
18S
28S
18S
28S

Figure 5-3C Sucrose gradient analysis of ^{3}H-uridine labelled total RNA from previtellogenic ovary

This sedimentation analysis was performed exactly as described in Figure 5-3A except that the total RNA samples were from the experiment described in Figure 5-1C and were labelled with ^{3}H-uridine. Recovery of loaded cpm from the gradients was over 75% and the data is expressed in the same manner as in Figures 5-3A and B. The following cpm were recovered:

17 hours 9,200 cpm;	30 hours 13,200 cpm;
48 hours 23,100 cpm;	65 hours 40,600 cpm.

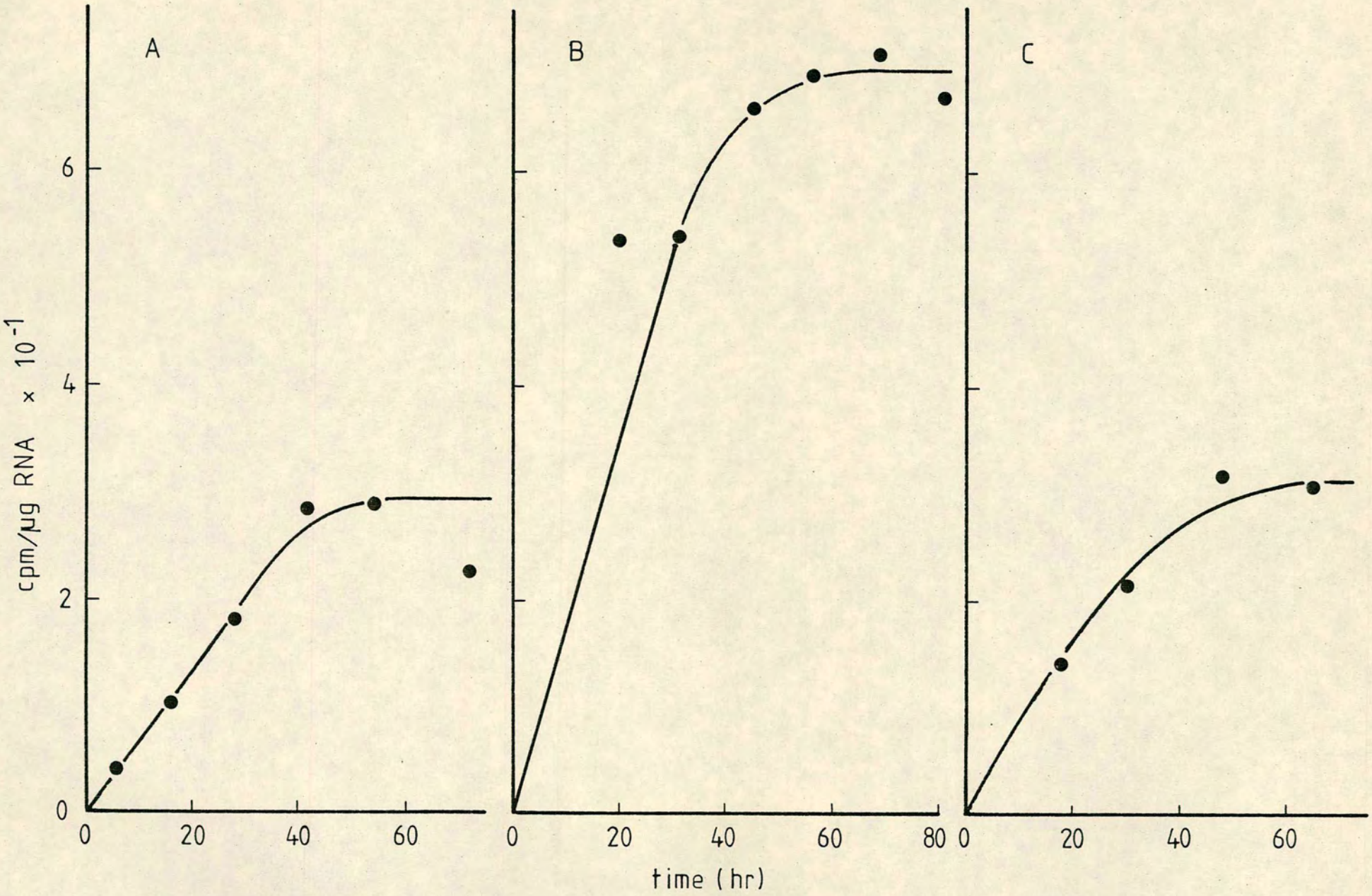

A
B
C
cpm/µg RNA × 10⁻¹
0
2
4
6
0
20
40
60
80
time (hr)

Figure 5-4 Incorporation kinetics for oligo (dT) bound RNA in previtellogenic ovary

(A) Incorporation of ^{3}H-adenosine

The total RNA extracted in the experiment described in Figure 5-1A was dissolved in 0.25 ml of binding buffer and oligo (dT) bound RNA was extracted as described in materials and methods section (h). Samples of the oligo (dT) bound fractions were precipitated with TCA and the radioactivity determined. The results are expressed as cpm/μg total RNA extracted. Recovery of loaded cpm after oligo (dT)-cellulose chromatography was over 96%.

(B) Incorporation of ^{3}H-adenosine

The oligo (dT) bound RNA was prepared exactly as described in Figure 5-4Ausing the total RNA samples from the experiment described in Figure 5-1B and the radioactivity incorporated is expressed in the same way as in Figure 5-4A.

(C) Incorporation of ^{3}H-uridine

The data for incorporation of ^{3}H-uridine into oligo (dT) bound RNA was obtained in the manner outlined in Figures 5-4A and B, but refers to samples prepared from total RNA from the experiment described in Figure 5-1C.

The oligo (dT) bound RNA synthesized by these ovaries was analysed on sucrose gradients and the results are given in Figure 5-5 from which the following observations can be made.

The newly labelled oligo (dT) bound RNA sediments in a broad heterogeneous manner and has a mean value about 22S. In all the gradients but especially at early times, a significant proportion (10-25%) of the RNA pellets during the run. Most of the gradients are relatively free of 4S/5S, 18S and 28S RNA contamination and there is certainly no systematic contamination observed. Consequently, no correction need be applied to the kinetic curve (Figure 5-4) as was the case for stage 6 oocyte oligo (dT) bound RNA (Chapter 4). Since at early times a larger proportion of this oligo (dT) bound RNA ($poly(A)^+RNA$) pellets under these conditions it may be that these molecules are synthesized as larger precursors, although in the steady state there is still about 10% of the incorporation into oligo (dT) bound RNA in molecules which pellet. The sedimentation profiles are similar to those obtained by labelling *in vivo* (Ford et al., 1977).

The fact that these experiments were performed using total ovary means that the follicle cells surrounding the oocytes will be contributing to the overall RNA synthesis observed. The extent of their contribution, in terms of total RNA synthesis, must be fairly small since it is known that previtellogenic oocytes of *Xenopus laevis* synthesize a large amount of 4S and 5S RNA and very little 18S and 28S RNA (Ford, 1972; Thomas, 1974; Ford et al, 1977). The patterns of total RNA synthesis described here are in agreement with this observation. However, for oligo (dT) bound RNA synthesis the follicle cells could be contributing disproportionately more, and therefore an attempt was made to remove them prior to RNA extraction. This is described in the next section.

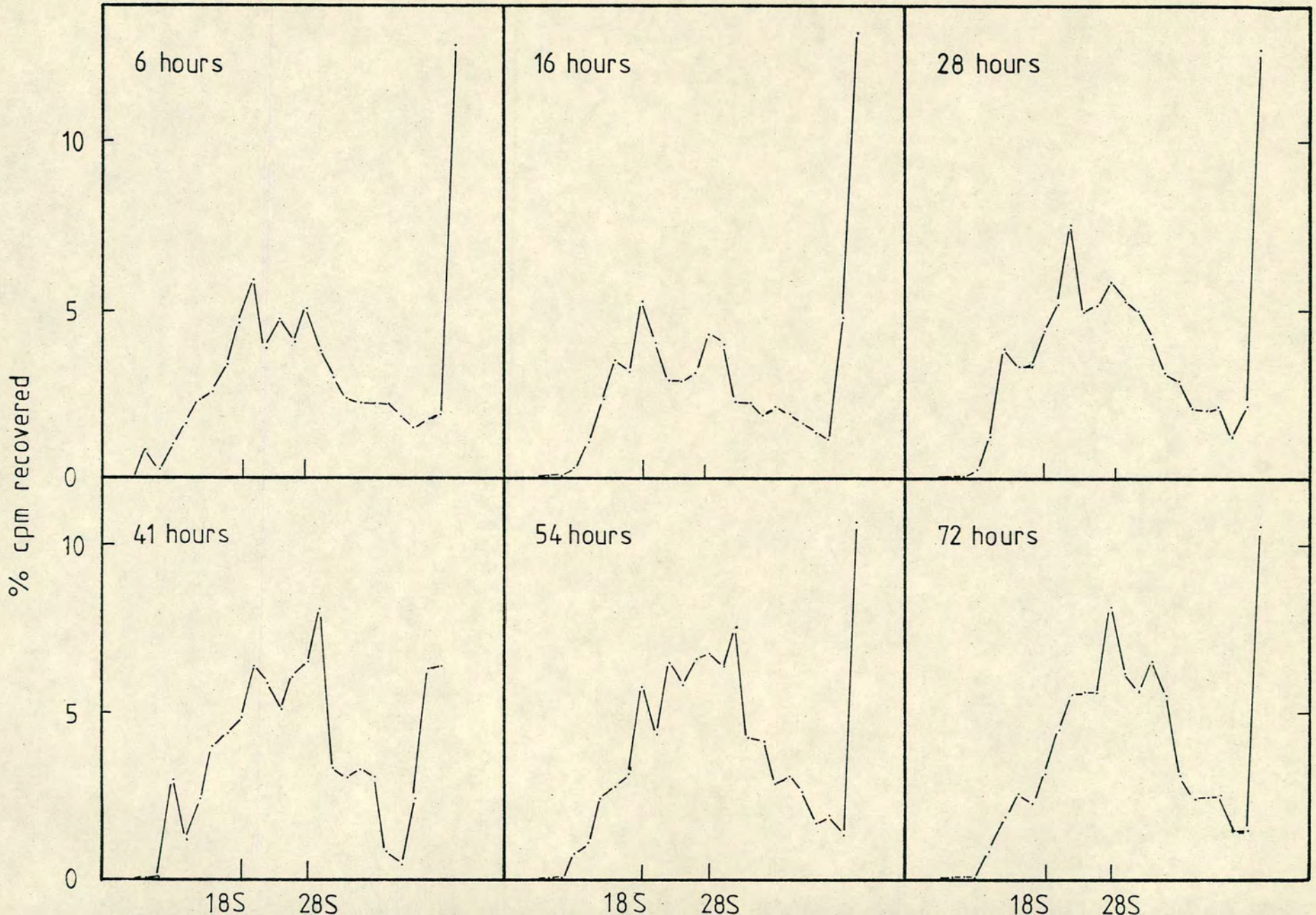

6 hours
16 hours
28 hours
41 hours
54 hours
72 hours
% cpm recovered
10
5
0
18S
28S

Figure 5-5A Sucrose gradient analysis of ^{3}H-adenosine labelled oligo (dT) bound RNA from previtellogenic ovaries of Xenopus laevis

Samples of oligo (dT) bound RNA prepared from the total RNA of the experiment described in Figure 5-1A were dissolved in 0.25 ml NETS and sedimented in 11.5 ml linear 7-30% sucrose gradients in NETS buffer at 40,000 rpm for 4½ hours at 25°C in an MSE 6 x 14 ml Ti rotor. The gradients were analysed as described in materials and methods section (i). Recovery of loaded cpm was over 85% and the figures show the percentage of total recovered ^{3}H cpm which is in each fraction. Centrifugation was from left to right, the last fraction is the pellet and the positions of the 18S and 28S RNA are marked. The following cpm were recovered:

6 hours 4,400 cpm; 16 hours 3,800 cpm; 28 hours 3,650 cpm
41 hours 2,100 cpm; 54 hours 3,700 cpm; 72 hours 3,400 cpm.

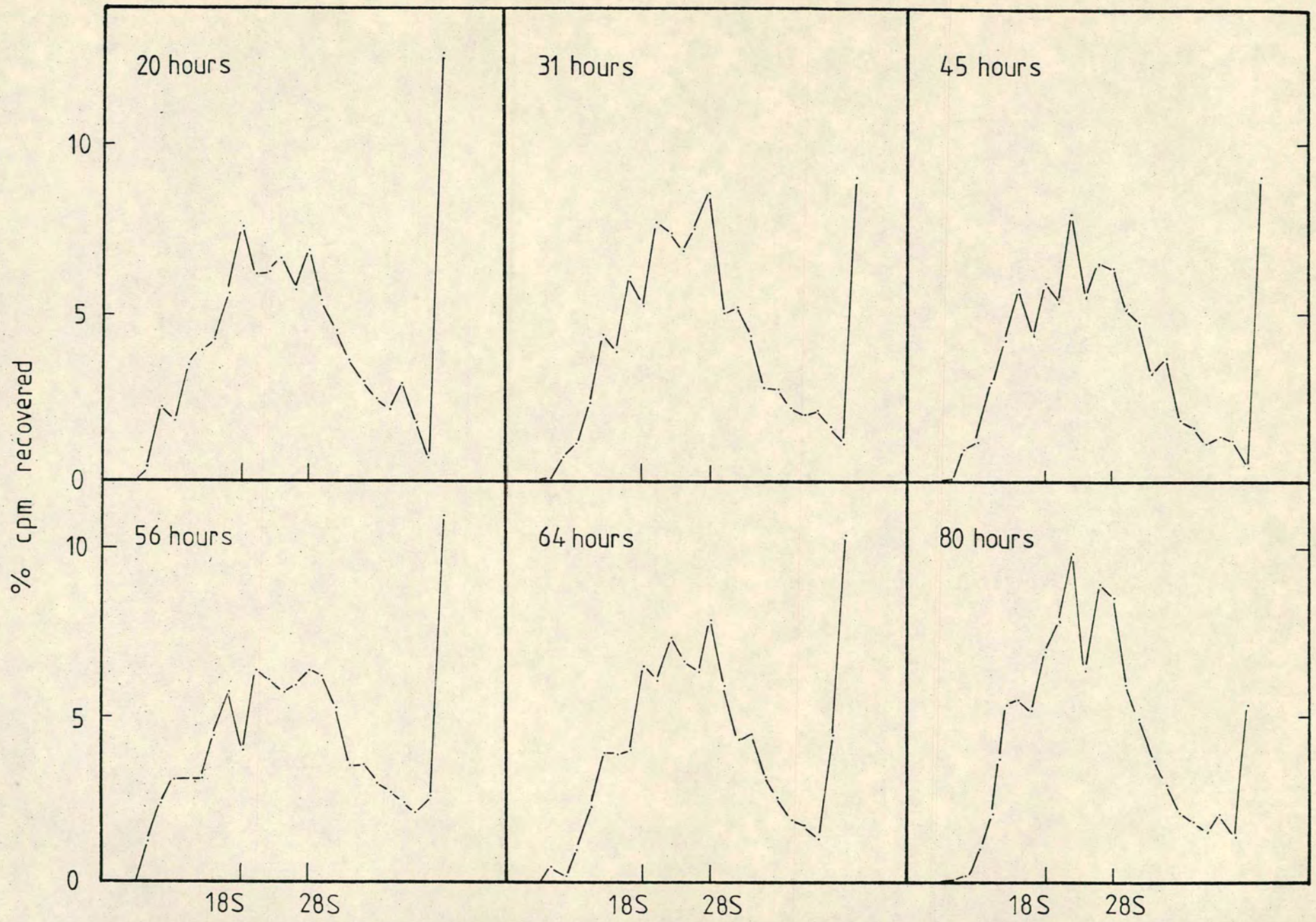

20 hours
31 hours
45 hours
56 hours
64 hours
80 hours
% cpm recovered
10
5
0
18S
28S

Figure 5-5B Sucrose gradient analysis of ^{3}H-adenosine labelled oligo (dT) bound RNA from previtellogenic ovaries of Xenopus laevis

These gradients were run in an identical manner to those described in Figure 5-5A but the samples of oligo (dT) bound RNA were prepared from the total RNA of the experiment described in Figure 5-1B. Recovery of loaded cpm was over 85%. The following cpm were recovered:

20 hours 5,250 cpm; 31 hours 5,500 cpm; 45 hours 3,600 cpm;

56 hours 5,100 cpm; 64 hours 5,800 cpm; 80 hours 5,200 cpm.

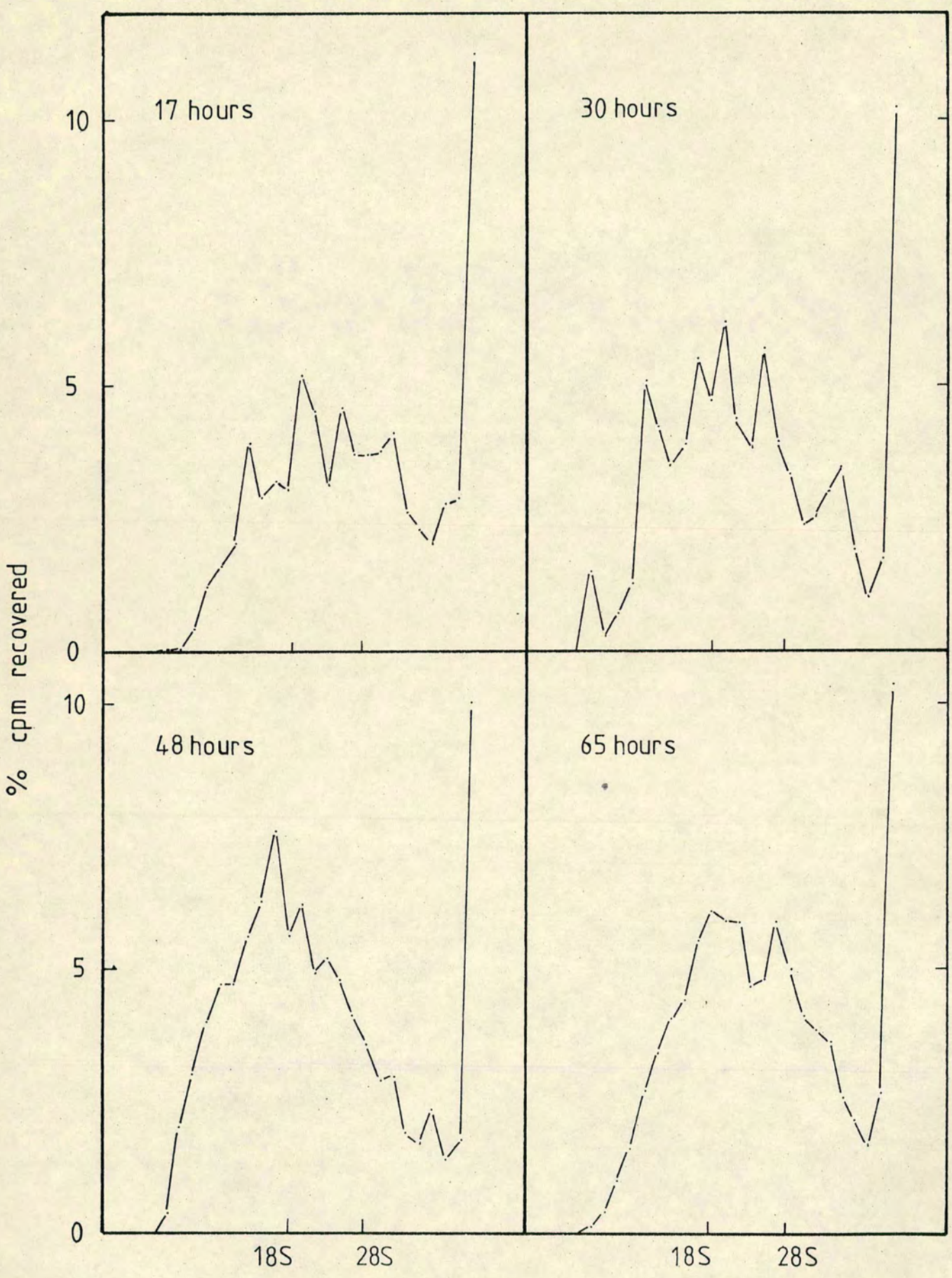

17 hours
30 hours
48 hours
65 hours
10
5
0
% cpm recovered
18S
28S

Figure 5-5C Sucrose gradient analysis of ^{3}H-uridine labelled oligo (dT) bound RNA from previtellogenic ovaries of Xenopus laevis

These gradients were run in an identical manner to those described in Figure 5-5A but the samples of oligo (dT) bound RNA were prepared from the total RNA of the experiment described in Figure 5-1C. Recovery of loaded cpm was over 80%. The following cpm were recovered:

17 hours 6,000 cpm; 30 hours 4,100 cpm;

48 hours 9,000 cpm; 65 hours 11,100 cpm.

(c) Defolliculated previtellogenic ovary

The method of removing follicle cells from previtellogenic ovary is detailed in materials and methods section (e). It involves the combined action of the enzymes collagenase and hyaluronidase and only sufficient agitation to free the oocytes from the connective tissue stroma should be applied otherwise disruption of the oocytes occurs. The method seems also to be very dependent on the ovaries chosen since some, perhaps those in which the oocytes are a little larger, seem to give a higher yield than do other ovaries. Also, in some cases the oocytes are freed but still have some follicle cells attached. Therefore it is necessary to examine the freed oocytes under the microscope and reject any preparations which are too impure. A further problem is that in some preparations the oocytes, although relatively free of follicle cells, are contaminated with a number of broken oocytes and germinal vesicles. Preparations of this type lead to scatter in the time points. In the experiments which follow the ovarian material not included in the oocyte preparation was also processed. By microscopic examination this fraction still contained many oocytes (intact and broken) as well as the follicle cells and stroma.

Preliminary experiments which separated the previtellogenic oocytes prior to their incubation showed that survival of these isolated oocytes over long periods was not good. It was therefore decided that this method of follicle cell removal should be applied after incubation of the ovary. Despite all these difficulties the method is useful since it gives information about oocytes alone and not whole ovary.

Incorporation into total RNA in previtellogenic oocytes

Figure 5-6 presents the kinetics of incorporation of radioactive uridine into total RNA by previtellogenic oocytes. Also presented are the same results expressed in cpm/ μg RNA isolated and the incorporation into the remainder of the ovary again expressed as cpm/ μg RNA. The recovery of RNA and oocytes in each of the samples is given and from this data the following observations can be made:

(1) Incorporation of uridine into previtellogenic oocytes is essentially linear at least up to 70 hours of incubation showing that stable RNA issynthesized.

(2) Incorporation into the remainder of the ovary is also linear, meaning that stable RNA is being made in this fraction.

(3) There is less incorporation per μg RNA into the oocytes than there is into the remainder of the ovary. This result must reflect the fact that the "remainder" fraction is enriched for follicle cells which are unlikely to have large cellular stores of RNA and thus the cpm/ μg RNA should be greater than for oocytes. If this explanation is correct, then the separation must have been successful.

(4) In most samples less than 50% of the recovered RNA is in the oocyte fraction. This suggests that isolated previtellogenic oocytes are less stable in the enzyme medium than oocytes still in the ovary.

(5) The overall recovery of RNA is low compared to previous experiments not using this separation procedure and therefore a substantial number of oocytes must have been disrupted during the enzyme treatment.

(6) The RNA content of these oocytes was about 0.06 μg/oocyte, and their diameters were consistent with stage 1 oocytes (Table 1-1).

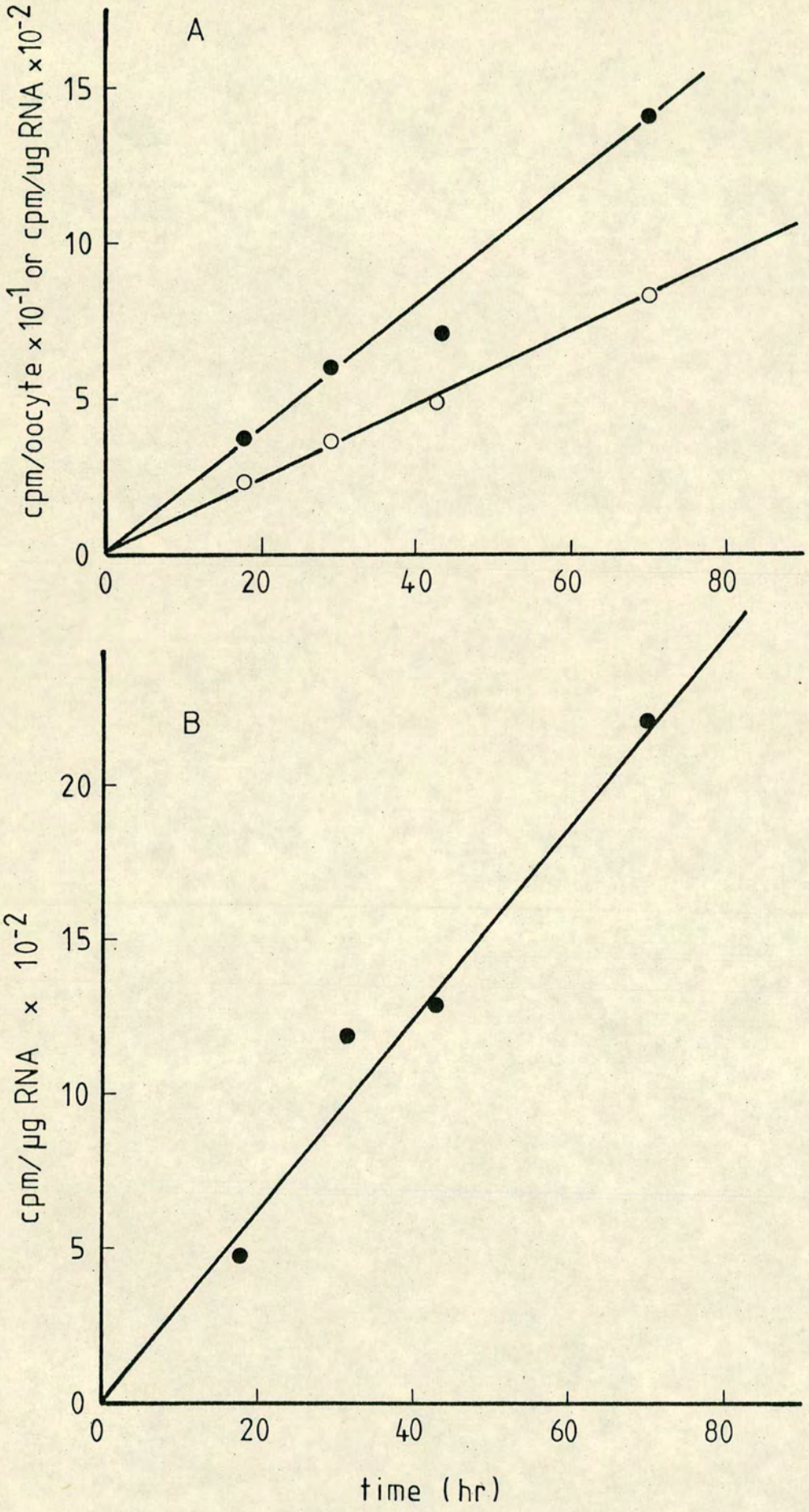

A
cpm/oocyte x 10^-1 or cpm/ug RNA x 10^-2
0
5
10
15
0
20
40
60
80
B
cpm/µg RNA x 10^-2
0
5
10
15
20
0
20
40
60
80
time (hr)

Figure 5-6 Incorporation kinetics for total RNA synthesis *in vitro* in previtellogenic oocytes of *Xenopus laevis*

5 immature ovaries were incubated as described in materials and methods section (c) in MBX containing 350 μCi/ml of 5-^{3}H-uridine. At the times indicated approximately 1/6 of the ovarian material was selected and the follicle cells were removed by the enzyme treatment detailed in materials and methods section (e). The total number of oocytes in each preparation was determined by counting the number present in a sample of the preparation and the values are given below. Samples of homogenate were dried onto GF/C filters, the radioactivity determined and RNA was extracted from the isolated oocytes and also from the remainder of the ovary. The optical densities of samples of total RNA were measured in a Unicam SP 1800 spectrophotometer. Samples of total RNA were precipitated with TCA and the radioactivity determined.

Time Point	Oocyte Samples				Remainder of ovary	
	No. of oocytes	RNA content (μg)	RNA content (ng/oocyte)	Soluble cpm per μg RNA	RNA content (μg)	Soluble cpm per μg RNA
18 hours	950	62	65	1,297	74	1,262
29 hours	1,200	72	60	1,105	91	1,379
43 hours	350	20	57	1,129	56	1,188
70 hours	600	35	58	1,218	37	1,045

(A) In the figure the results are expressed as cpm of ^{3}H-uridine incorporated per oocyte (O) and cpm incorporated per μg of total RNA recovered from the oocytes (●).

(B) ^{3}H-uridine incorporation into the remainder of the ovary. The results are expressed as cpm incorporation per μg total RNA recovered.

Samples of the total RNA synthesized by the oocyte and the "remainder" fraction were analysed on sucrose gradients and the results are given in Figure 5-7. At all time points the gradient profiles of oocyte total RNA are similar in that there is predominently a single large peak of radioactivity sedimenting in the region 4S/5S. Traces of 18S and 28S RNA are seen on a small heterogeneous background. The average distribution of radioactivity for the oocyte fraction is 82% 4S/5S RNA, 6% 18S and 28S RNA and 12% heterogeneous RNA. This agrees well with other data (Thomas, 1974).

For the "remainder" fraction similar gradient profiles are obtained but the proportion of radioactivity in 18S and 28S RNA is considerably greater. The average distribution of radioactivity in this fraction is 66% 4S/5S RNA, 19% 18S and 28S RNA and 15% heterogeneous RNA.

It is thus possible to conclude that the contamination of the oocyte fraction by follicle cells is minimal. Even if all the labelled 18S and 28S RNA present is due to follicle cell synthesis the percentage contamination of oocyte RNA by follicle cell RNA is only 7.5% (assuming 80% of follicle cell RNA synthesis is 18S and 28S RNA). In this connection Thomas (1974) found that less than 3% of the labelled cytoplasmic RNA of stage 1 oocytes was rRNA. Contamination of the "remainder" fraction by oocytes is much heavier. In fact most (over 75%) of this newly labelled RNA must be oocyte in origin (assuming 15% of the follicle cell RNA synthesis is 4S/5S RNA).

Incorporation into oligo (dT) bound RNA in previtellogenic oocytes

Figure 5-8 shows the kinetics of incorporation of radioactive uridine into oligo (dT) bound RNA by previtellogenic oocytes and by the remainder of the ovary. Despite the very large scatter in the points

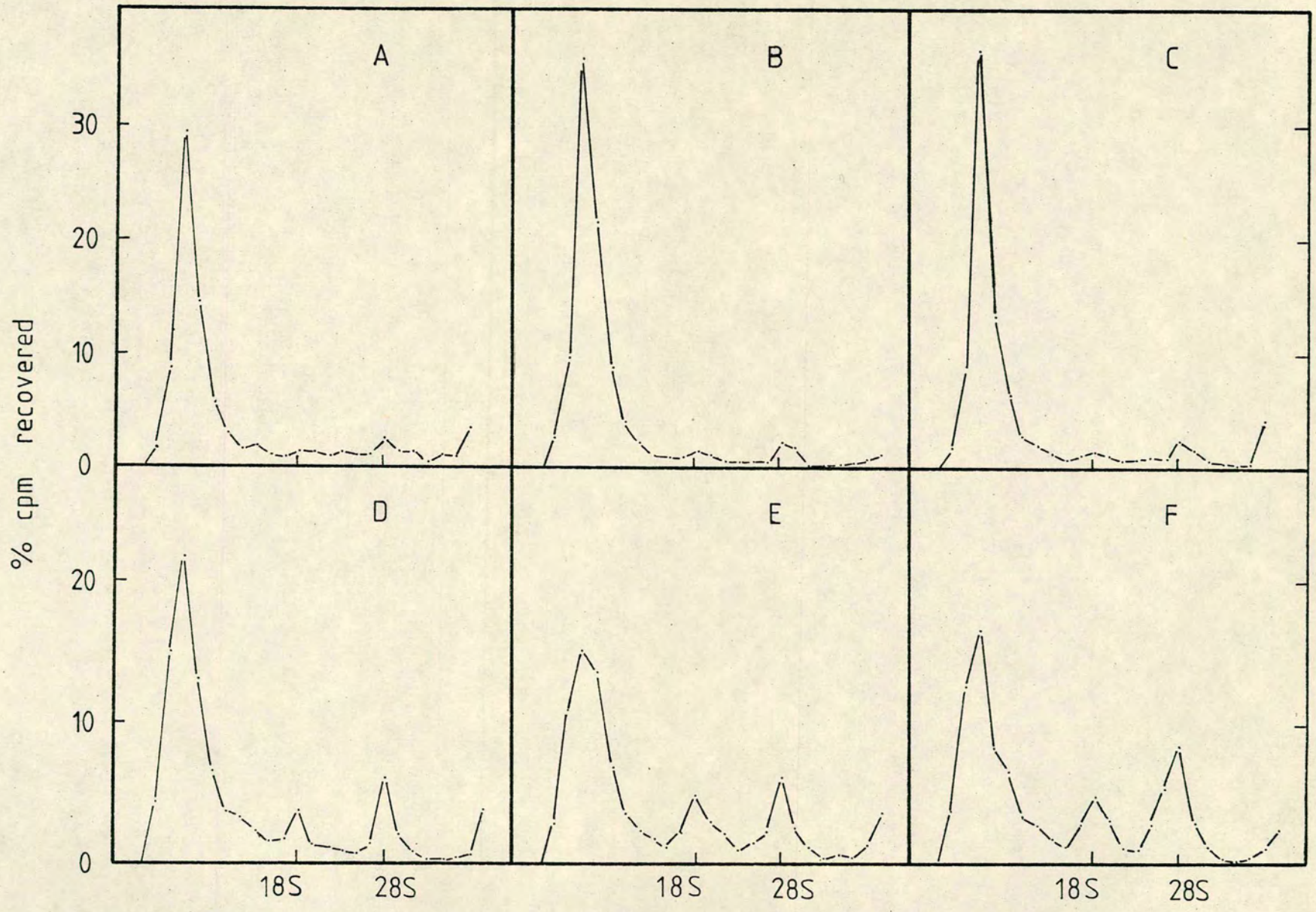

A
B
C
D
E
F
30
20
10
0
20
10
0
% cpm recovered
18S
28S
18S
28S
18S
28S

Figure 5-7 Sucrose gradient analysis of *in vitro* labelled total RNA from previtellogenic oocytes and ovary of *Xenopus laevis*

Samples of total RNA from the experiment described in Figure 5-6 were sedimented in 11.5 ml linear 7-30% sucrose gradients in NETS at 40,000 rpm for 4½ hours at 25°C in an MSE 6 x 14 ml Ti rotor. The gradients were analysed as described in materials and methods section (i). Recovery of loaded cpm was over 70%. The following cpm were recovered:

Previtellogenic oocytes

A = 29 hours 3,900 cpm;
B = 43 hours 1,150 cpm;
C = 70 hours 4,300 cpm.

Remainder of ovary

D = 29 hours 10,800 cpm;
E = 43 hours 8,000 cpm;
F = 70 hours 8,300 cpm.

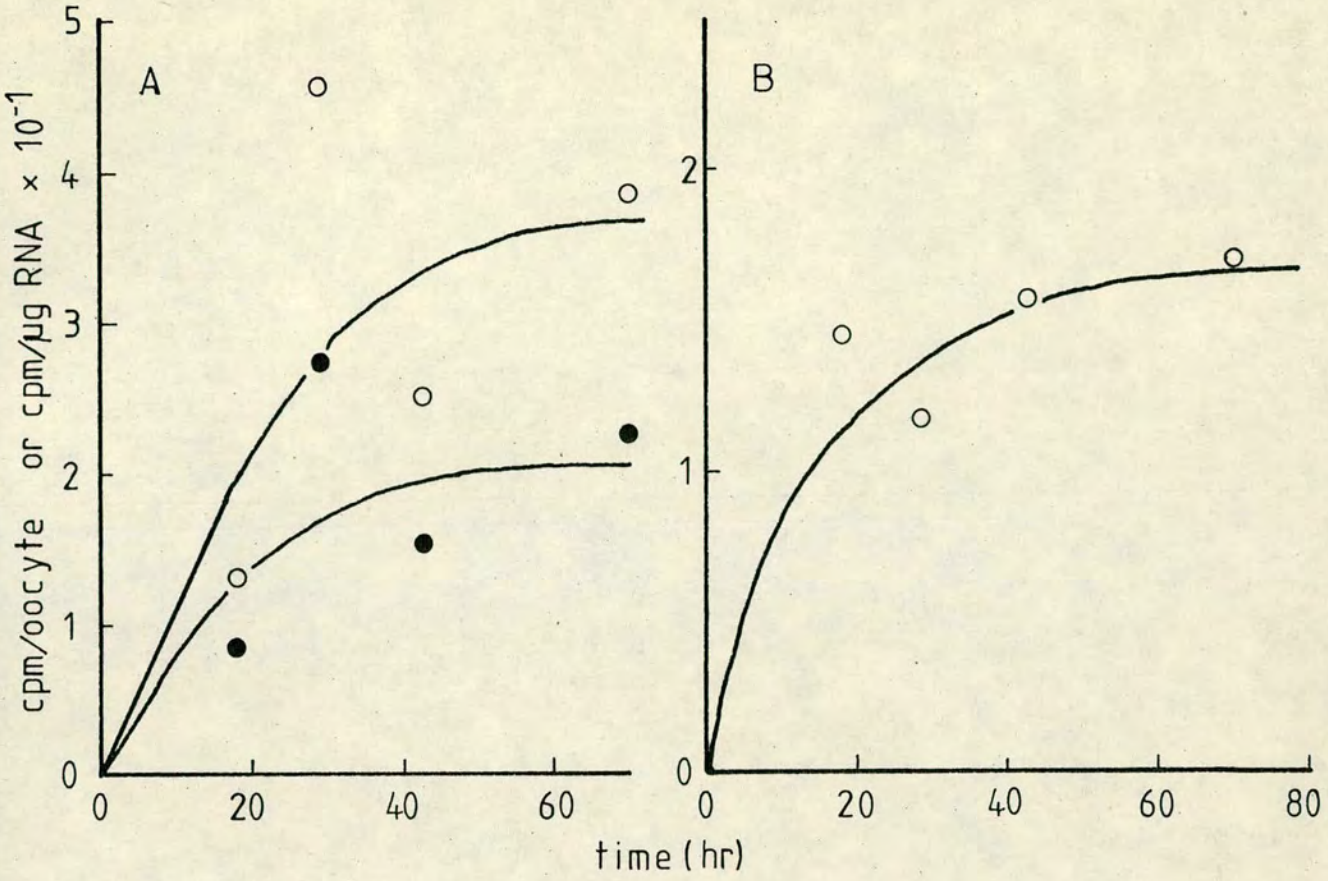


Figure 5-8 Incorporation kinetics for oligo (dT) bound RNA synthesis in vitro in previtellogenic oocytes of Xenopus laevis

Oligo (dT) bound RNA was prepared from ^{3}H-uridine labelled total RNA samples of the experiment described in Figure 5-6 and the entire samples were precipitated with TCA and the radioactivity determined.

(A) Incorporation into oligo (dT) bound RNA in previtellogenic oocytes. The incorporation is expressed as cpm per oocyte (●) and cpm per µg total RNA recovered (○).

(B) Incorporation into oligo (dT) bound RNA in the remainder of the ovary. The incorporation is expressed as cpm per µg total RNA recovered.

the data is not inconsistent with that presented in the previous section for intact ovary. The curve for the remainder of the ovary definitely does saturate, however it is not known what proportion of this labelled oligo (dT) bound RNA is due to follicle cell synthesis.

It is possible to make an estimate on the basis of the 4S/5S RNA contamination observed in the total RNA in the "remainder" fraction. If it is assumed that the proportions of total RNA synthesis devoted to the various RNA classes in follicle cells are similar to other somatic cells and if it is further assumed that all the 18S and 28S RNA synthesis observed in both the ovary fractions is due to follicle cell synthesis, then the following calculations can be made. Follicle cell RNA synthesis is assumed to be 15-20% 4S/5S RNA, 75% 18S and 28S RNA and 5% mRNA. The observed incorporation into classes of RNA in the remainder of the ovary is 66% 4S/5S RNA, 19% 18S and 28S RNA, 15% heterogeneous RNA and 3% mRNA. Therefore, the maximum amount of 4S/5S RNA due to follicle cells in the remainder of the ovary is $20\% \div 75\% \times 19\% = 5\%$. Therefore the minimum amount of 4S/5S RNA synthesis in the "remainder" fraction due to oocytes is $(66-5) \div 66 = 90\%$. One might therefore expect that over 90% of the mRNA synthesis is due to oocyte synthesis in this fraction. This calculation can only be approximate but it is more likely to underestimate the proportion of mRNA synthesis due to oocytes since it assumes that all the 18S and 28S RNA synthesis is due to the follicle cells.

This estimate then supports the idea that the kinetic curve for oligo (dT) bound RNA synthesis in the remainder of the ovary is largely due to the oocytes present, and thus permits the conclusion that the same curve for the oocytes alone does saturate even though a straight line could be drawn through the scattered data points

(Figure 5-8). It is thus concluded that incorporation of uridine into oligo (dT) bound RNA in previtellogenic oocytes saturates and therefore if the specific activity is constant the oligo (dT) bound RNA is unstable and turns over with a half-life of 10-15 hours. An alternative explanation could be that the oligo(dT) bound RNA synthesis and not total RNA synthesis is dependent on some factor which runs out after 30-40 hours of incubation in vitro. This alternative was considered unlikely since it is not applicable in the case of 4 day old stage 6 oocytes which give the same kinetic curve for oligo (dT) bound RNA synthesis as do fresh stage 6 oocytes.

The very low recovery of isolated oocytes and hence oocyte oligo (dT) bound RNA made sucrose gradient analysis of this RNA very difficult. The best gradient data obtained was consistent with that presented in the previous section for previtellogenic ovary oligo (dT) bound RNA. In the case of previtellogenic ovary however there was sufficient incorporation of radioactive adenosine into oligo (dT) bound RNA to permit the sizing of the newly labelled poly(A) on SDS/acrylamide gels. The results are presented in the next section.

(d) Poly(A) sizes of previtellogenic ovary oligo (dT) bound RNA

Oligo (dT) bound RNA samples from previtellogenic ovary labelled with adenosine were pooled and digested with RNAse T_1 and RNAse A, with melting half way through the digestion. The RNA was re-extracted from the digest and run on standard poly(A) sizing gels (materials and methods, section (1)). The results of this procedure for two different batches of adenosine labelled oligo (dT) bound RNA are given in Figure 5-9. The two results which are remarkably similar show that newly labelled previtellogenic ovary poly(A) is quite homogeneous

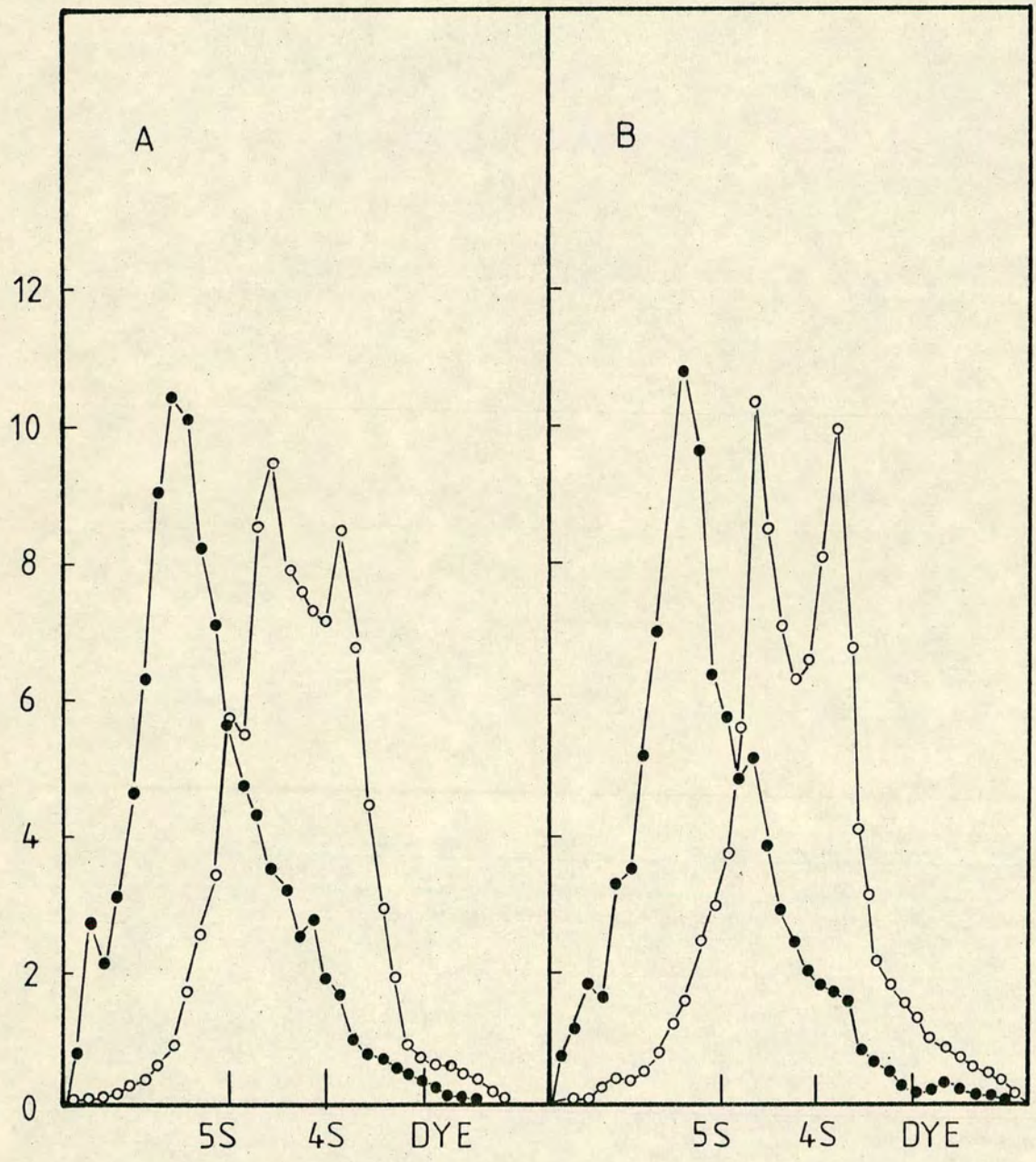

A
B
12
10
8
6
4
2
0
5S
4S
DYE
5S
4S
DYE

Figure 5-9 Poly(A) size distribution in newly labelled and steady state oligo (dT) bound RNA from previtellogenic ovary of Xenopus laevis

Poly(A) was prepared from pooled samples of ^{3}H-adenosine labelled oligo (dT) bound RNA from the experiments described in Figures 5-4 and 5-5 as described in materials and methods section (j). The poly(A) was analysed on 10% SDS/acrylamide gels as described in materials and methods section (l). The poly(A) was eluted from the gel slices and aliquots (10 µl ex 1.2 ml) were taken for hybridization with ^{3}H-poly(U) as described in section (k) of materials and methods and the remaining poly(A) was precipitated with TCA. Recovery from the gels was over 80%. The position of the following markers, run on a parallel gel, are shown from left to right 5S RNA, 4S RNA and bromophenol blue. Using the calibration curve derived by Cabada et al. (1977) these markers correspond to poly(A) with the following number of A residues:

5S RNA (82); 4S RNA (57); bromophenol blue (38).

(A) RNA from the experiment described in Figure 5-4A

(•——•) Incorporation of ^{3}H-adenosine into poly(A); percentage of 19,200 cpm recovered.

(o——o) ^{3}H-poly(U) binding to total (steady state) poly(A); percentage of 40,100 cpm recovered.

(B) RNA from the experiment described in Figure 5-4B

(•——•) Incorporation of ^{3}H-adenosine into poly(A); percentage of 19,700 cpm recovered.

(o——o) ^{3}H-poly(U) binding to total (steady state) poly(A); percentage of 24,600 cpm recovered.

with a mean size of about 100 A residues. In each case the steady state poly(A) size profile obtained by poly (U) hybridization is given for comparison and it is clear that some newly synthesized poly(A) molecules are larger than the steady state size. It must be pointed out that the labelled poly(A) comes from pooled time points in the range 10 to 80 hours, therefore it is possible that changes in the newly labelled poly(A) size profile might have been observed if early and late time points had been compared. Since, in each of these two experiments at least half of the time points were after saturation of incorporation into poly(A)$^+$RNA had occurred, the absence of much labelled poly(A) of the steady state size must mean that if the newly labelled poly(A) is eventually processed to the steady state size then the processing time is quite lengthy. The alternative explanation is that the newly labelled molecules do not enter the pool of steady state molecules and therefore their poly(A) is not processed to the steady state size.

As explained in materials and methods section (p) it is possible to convert the TCA precipitable cpm in each gel slice into molecules per gel slice providing the specific activity of the poly(A) and also the number of A residues per gel slice are known. If the specific activity is taken to be 100 cpm/pmole (Chapter 6) and the number of molecules of each size class calculated, then the result is given in Figure 5-10 from which it can be seen that half the labelled poly(A) molecules have a size less than 90 A residues. Labelled molecules of the steady state size are present and may account for a third of the total number of molecules. With the results plotted in this way the possibility that the newly synthesized previtellogenic ovary poly(A) shortens to the steady state size within

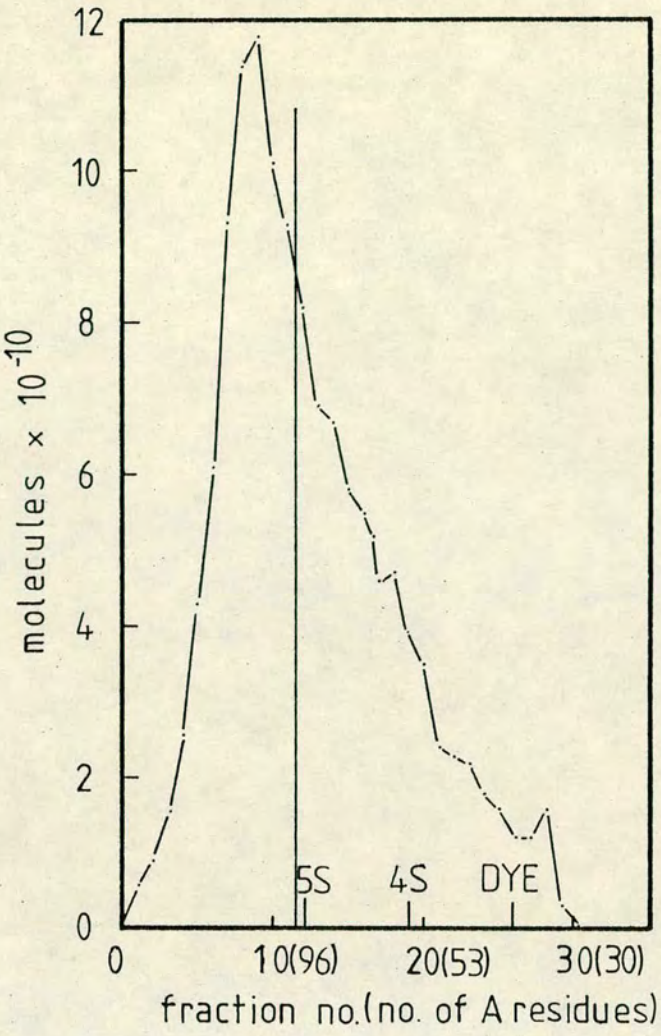


Figure 5-10 Distribution of poly(A) size on a molecular basis

The poly(A) size distribution shown in Figure 5-9A was converted to the number of molecules of each poly(A) size using the method outlined in materials and methods section (p). The specific activity of the radioactive poly(A) was assumed to be 100 cpm/pmole since a value of 105 cpm/pmole was measured in a similar experiment in Table 6-5. From left to right the positions of 5S RNA, 4S RNA and bromophenol blue are marked and the line corresponds to the number average size of the poly(A) molecules. The abcissa is calibrated in both fraction number and corresponding poly(A) size in brackets.

20-40 hours seems more likely, but since time points were pooled and the poly(A) processing events in oocytes and follicle cells probably differ, this is simply speculation.

Using 10,000 as the number of oocytes in a previtellogenic ovary (Ford, personal communication) the number of labelled molecules is calculated to be 4×10^7 per previtellogenic oocyte (and follicle). The number of labelled poly(A) molecules calculated in this manner constitutes about 0.2% of the steady state number obtained by poly (U) hybridization, in previtellogenic ovary (Cabada et al., 1977), but is about an order of magnitude fewer than are labelled in the stage 6 oocyte (Chapter 4).

(e) Discussion

In attempts to study RNA synthesis in previtellogenic oocytes the technique of microinjection with its advantages could not be applied due to the small size of these oocytes. Consequently previtellogenic ovaries were incubated in ^{3}H-nucleoside and TCA precipitable cpm incorporated into various classes of RNA were monitored. RNA was made in previtellogenic ovary which was stable over the 80 hours studied and which was about 50-60% 4S/5S, 20-25% 18S and 28S and 20-25% heterogeneous RNA. These observations are consistent with earlier work (Ford, 1972; Rosbash & Ford, 1974; Thomas, 1974; Ford et al., 1977).

Using a fairly difficult procedure RNA synthesis in previtellogenic oocytes devoid of their follicle cells was studied, and for total RNA synthesis the results showed that stable RNA was made which had the following composition: 82% 4S/5S RNA, 6% 18S and 28S RNA and 12% heterogeneous RNA. It is possible that the incorporation into 18S and 28S RNA was due to follicle cells which contaminated the oocyte

preparation although some basal synthesis of 18S and 28S RNAs may exist in these stage 1 oocytes prior to the massive activation which occurs at about the beginning of vitellogenesis (Ford, 1972).

Incorporation into oligo (dT) bound RNA in previtellogenic ovary accounted for about 5% of the incorporation into total RNA until about 30-40 hours when the former incorporation levelled off. Using isolated oocytes a stong case can be made for saying that the incorporation into oligo (dT) bound RNA in total ovary was due mainly to oocyte and not follicle cell oligo (dT) bound RNA synthesis. The possibility that there was a stable component in the oligo (dT) bound RNA fraction has not been ruled out, but seems unlikely.

The newly synthesized poly(A) size in previtellogenic ovary was shown to be about 90 A residues, which was longer than the characteristic steady state profile, however molecules of the steady state size were present and since the experimental sample was pooled early and late time points, the concept that the newly synthesized poly(A) might shorten to the steady state size before the whole molecule is turned over cannot be ruled out. An indirect calculation based mainly on assumptions about the precursor specific activity showed that the number of newly labelled poly(A) molecules was approximately 0.1% of the steady state number obtained using the poly(U) binding assay.

Table 5-1 compiles the rates of incorporation of nucleosides into RNA in previtellogenic ovary. In four different experiments the rate of incorporation of nucleoside into total RNA varies less than two fold (if allowance is made for the lower radioactive concentration in experiment 1), and allowing for base composition differences between incorporation of ^{3}H-adenosine and ^{3}H-uridine into oligo (dT) bound RNA,

Table 5-1 Comparison of rates of incorporation of radioactive nucleoside into stable RNA and oligo (dT) bound RNA

		A	B	C	D
Exp.	Label	Total RNA stable rate of incorporation	Oligo (dT) bound RNA Initial Rate of incorpn.	Oligo (dT) bound RNA steady state amount	Column B as % of Column A
	(μCi/ml)	cpm/μg RNA/hr	cpm/μg RNA/hr	cpm/μg RNA	%
1	^{3}H-adenosine (312)	12	0.7	29	5.7
2	^{3}H-adenosine (350)	29	1.8	70	6.3
3	^{3}H-uridine (350)	24	0.9	31	3.7
4	^{3}H-uridine (350)	20	0.7	39	3.5

Data compiled from several experiments of the type described in Figures 5-1 and 5-4. The stable rate of incorporation corresponds to the linear part of curves of the type in Figure 5-1. The initial rate of incorporation and the steady state amount correspond to the rate to the first time point and to the plateau respectively in curves of the type in Figure 5-4. Since no specific activity estimations were made the data is expressed as cpm/μg RNA isolated/hour (columns A and B) and cpm/μg RNA in column C. In column D the initial rate of incorporation into oligo (dT) bound RNA is expressed as a percentage of the stable rate of incorporation into total RNA

the rate of incorporation into this class of RNA is a constant proportion of the total in different experiments. Without an accurate knowledge of the intracellular precursor specific activity in these incorporation experiments it is not possible to compare the rates of RNA synthesis in previtellogenic oocytes with those in stage 6 oocytes, but it is possible at this stage to compare some of the characteristics of oligo (dT) bound RNA synthesis in these two oocyte stages:

(1) The kinetics of incorporation of radioactive precursors into oligo (dT) bound RNA of both oocyte stages are quite similar, with perhaps the stage 6 oocyte curve reaching a steady state a little earlier.

(2) The oligo (dT) bound RNAs made are both heterogeneous, but whereas stage 6 oocyte RNA has a mean size of about 18S, the previtellogenic RNA seems to contain more larger molecules, some of which pellet.

(3) The size of the labelled poly(A) in previtellogenic ovary is fairly homogeneous at about 90 A residues whereas the cytoplasmic stage 6 oocyte labelled poly(A) is very homogeneous in size at about 60 A residues.

These observations permit one to say that there are sufficient differences in the properties of the oligo (dT) bound RNA at these two stages of oogenesis to consider them as different classes of RNA, although it may still be that there is differential synthesis of the larger molecules of the same population of transcripts in previtellogenic oocytes with presumably a change in the poly(A) processing during oogenesis.

In view of the difficulties of RNA synthetic rate comparison between oocyte stages, between different animals, or indeed between this work and that of other investigators some considerable amount of time was spent trying to develop an assay system for measuring the precursor specific activity in oocyte extracts. This work is presented in the next Chapter.

CHAPTER 6

Measurement of Specific Radioactivities

(a) Introduction

In any study of RNA synthesis it is not possible to evaluate an absolute rate of synthesis without a knowledge of the radioactive precursor specific activity. In this particular investigation the comparison of rates of RNA synthesis in injected oocytes from different females has not been possible since specific radioactivities were not actually determined, but merely estimated on the basis of indirect measurements (Chapter 4). It follows that any differences between the estimated rates of RNA synthesis obtained in this study and in other investigations are likely to be due to inaccuracies in this method of estimating the initial specific radioactivity in injected oocytes. Furthermore the method of estimating specific radioactivity used for injected stage 6 oocytes is not at all applicable to oocytes which have been incubated in radioactive nucleoside. Consequently, it was not possible to compare rates of RNA synthesis in incubated previtellogenic oocytes (Chapter 5) and in injected stage 6 oocytes (Chapter 4).

It was therefore decided to attempt to develop a quick, generally applicable method of determining the specific radioactivity of precursor NTPs. Until recently the direct determination of specific radioactivities has been relatively difficult, often requiring large amounts of material. Even the sensitive enzymic methods such as the luciferase assay for ATP or GTP (Humphreys, 1973) or the hexokinase assay for ATP or UTP (Weigers et al., 1974) include time consuming chromatographic steps. Recently, a method of determining ATP or UTP pool size using the enzyme RNA polymerase has been described (Sasvari-Szekely et al., 1975) and it was hoped to be able to adapt this method for the measurement of both NTP pool

size and specific radioactivity. While this was in progress Maxson & Wu (1976) published a similar method in a general form.

(b) Theory

The initial experiments performed were based on the following logic. Using the standard E. coli RNA polymerase assay described in materials and methods section (o) to synthesize RNA in vitro on salmon sperm DNA template, if one of the NTP substrates is radio labelled (for example with ^{32}P-ATP) and its specific activity in cpm/pmole is known it follows that:

$$^{32}\text{P cpm in RNA} = \text{pmoles of RNA made} \times \frac{\text{cpm of } ^{32}\text{P-ATP present}}{\text{pmoles ATP present}}$$

If unradioactive ATP is added then:

$$^{32}\text{P cpm in RNA} = \text{pmoles of RNA made} \times \frac{\text{cpm of } ^{32}\text{P-ATP present}}{\text{pmoles ATP initially} + \text{pmoles ATP added}}$$

Therefore, by adding different known amounts of unlabelled ATP to a standard assay mix containing a constant amount of CTP, GTP, UTP and ^{32}P-ATP, it would be possible to construct a calibration curve from which the amount of unlabelled ATP added to the assay mix could be read off directly. This would be a determination of pool size if the ATP added was from a cell extract.

If, instead of adding unlabelled ATP, a quantity of radio labelled ATP is added, then providing the added ATP is labelled with a different radio-isotope (e.g. ^{3}H-ATP) it would be possible to calculate the original specific radioactivity of the added

radio labelled ATP as follows. The quantity of ATP added can be read off directly from the calibration curve giving for example, x pmoles of ATP added. Since both the amount and the initial specific radioactivity of the ^{32}P-ATP is known the final ^{32}P-ATP specific radioactivity (SA) in the assay mix is easily calculated:

$$\text{final SA of } {}^{32}\text{P-ATP in assay mix (cpm/pmole)} = \frac{\text{cpm of } {}^{32}\text{P-ATP present}}{\text{pmoles ATP initially in assay mix} + \text{x pmoles of ATP added}}$$

Then since the ratio of ^{32}P:^{3}H in the RNA product is equal to the ratio of specific radioactivities finally present in the assay mix, the final ^{3}H-ATP specific radioactivity in the assay mix can be calculated:

$$\text{final SA of } {}^{3}\text{H-ATP in assay mix (cpm/pmole)} = \text{final SA of } {}^{32}\text{P-ATP in assay mix (cpm/pmole)} \times \frac{{}^{3}\text{H cpm in RNA}}{{}^{32}\text{P cpm in RNA}}$$

and since the amount of ATP finally present in the assay mix is known it is possible to obtain the specific radioactivity of the added ^{3}H-ATP:

$$\text{SA of } {}^{3}\text{H-ATP added (cpm/pmole)} = \text{final SA of } {}^{3}\text{H-ATP in assay mix (cpm/pmole)} \times \frac{\text{pmoles ATP initially in assay mix} + \text{x pmoles ATP added}}{\text{x pmoles ATP added}}$$

This simplifies to:

$$\text{SA of } {}^{3}\text{H-ATP added (cpm/pmole)} = \frac{{}^{3}\text{H cpm in RNA}}{{}^{32}\text{P cpm in RNA}} \times \frac{\text{cpm of } {}^{32}\text{P-ATP present}}{\text{x pmoles of ATP added}}$$

Therefore providing the amount of ATP added (as ^{3}H-ATP) can be read off a calibration curve and the ratio of ^{3}H cpm:^{32}P cpm in the RNA product can be measured it is a simple matter to calculate the

specific radioactivity of the ^{3}H-ATP added to a standard assay mix with a known ^{32}P-ATP specific radioactivity. The method should obviously be generally applicable since by changing the labelled NTP in the assay mix any ^{3}H, ^{14}C or ^{32}P-NTP pool size and/or specific radioactivity could be determined.

(c) Optimization of the assay conditions

Initial experiments

A number of experiments were performed in order to optimize the assay system using salmon sperm DNA template and ^{3}H-ATP. Figure 6-1 presents data on the dependence of incorporation of ^{3}H-ATP into RNA on enzyme concentration. With the unlabelled NTP concentration in the assay mix at 0.05 mM and the ATP twice this, the enzyme concentration can be increased 10 to 15 times with an equal increase in incorporation. Further increases in enzyme concentration do not produce a linear increase in incorporation of ^{3}H-ATP into RNA, the stimulation of incorporation saturating at about a 30 times increase in enzyme concentration. The amount of incorporation is also known to be increased by the following factors (Sazvari-Szekely et al., 1975; Maxson & Wu, 1976; Kevin O'Hare, personal communication):

(1) An increase in NTP concentration.

(2) An increase in salt concentration up to 0.2 M.

(3) The time of incorporation.

(4) The specific radioactivity of the labelled NTP in the assay mix.

By considering these parameters the following assay mix was selected: unlabelled NTP 0.1 mM, 0.2 M KCl, 0.4 mM ATP at 0.5 x 10^{5} cpm/nmole, 10 units of enzyme, and incubation was carried out for 20 minutes. Figure 6-2 presents the results of an experiment on the

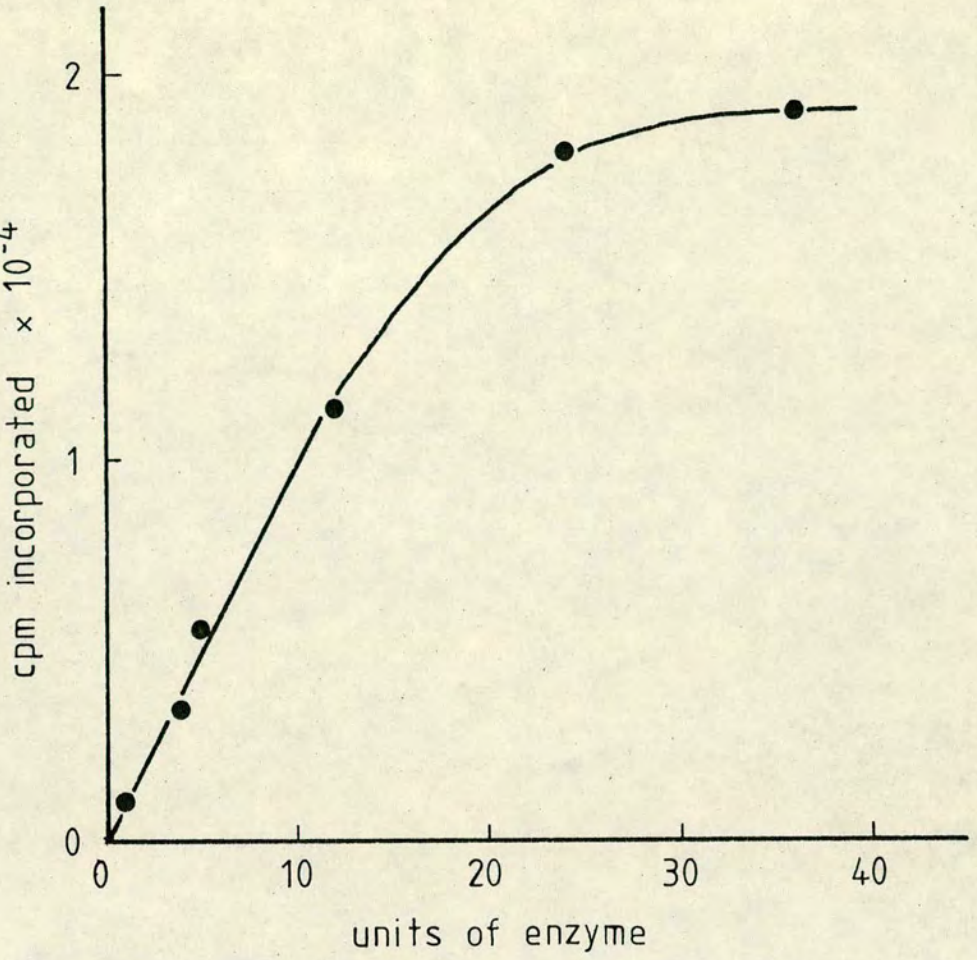


Figure 6-1 Effect of enzyme concentration on the incorporation of ^{3}H-ATP into RNA by E. coli RNA polymerase

RNA polymerase assays were carried out essentially as in Method 1, section (o) of materials and methods, but varying the amount of enzyme added while keeping the NTP and DNA concentrations constant. Negligible incorporation occured in the absence of enzyme. The data is plotted as TCA precipitable cpm incorporated versus units of enzyme added to the assay.

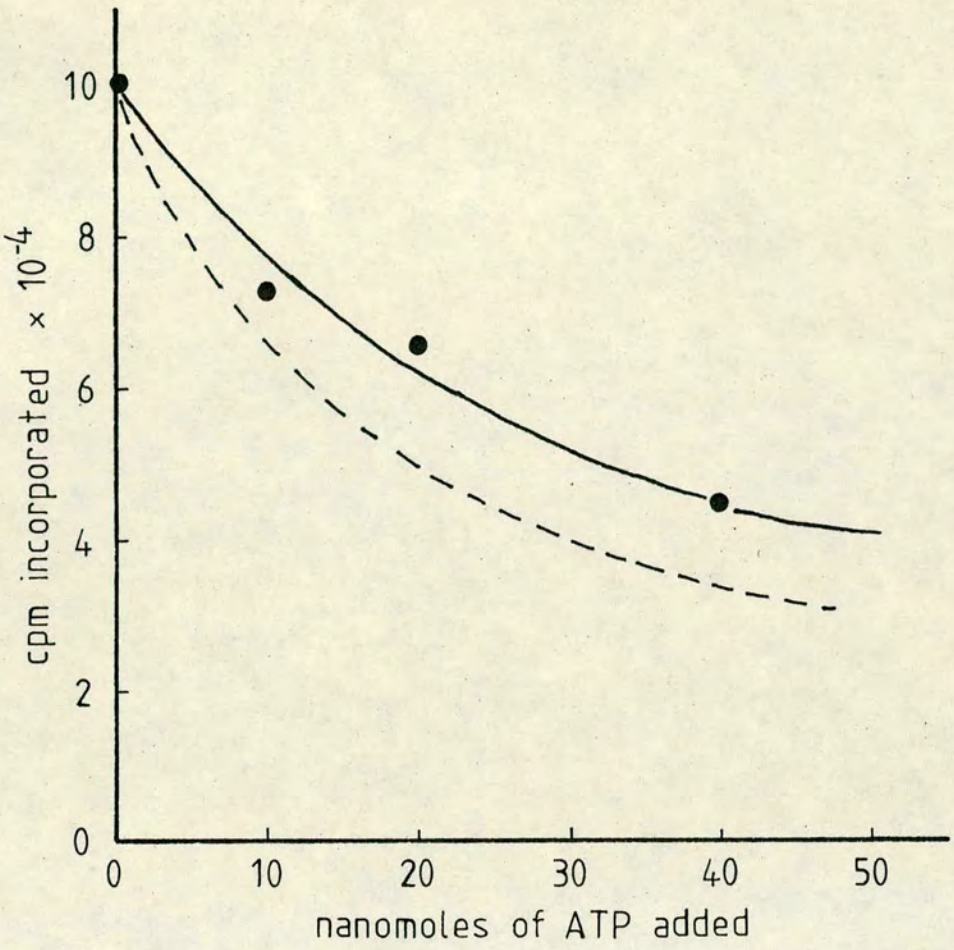


Figure 6-2 Effect of unlabelled ATP on the incorporation of ^{3}H-ATP into RNA by E. coli RNA polymerase

Using method 1 (section (o) of materials and methods), RNA polymerase assays were performed with 10 units of enzyme and additions of 0, 10, 20 and 40 nmoles of unlabelled ATP to the 20 nmoles ATP originally present. The data is plotted as TCA precipitable cpm incorporated versus nmoles of unlabelled ATP added. The dotted line represents the theoretical curve expected due to reduction of the specific radioactivity of the ^{3}H-ATP.

effect of adding unlabelled ATP to this assay mix. Since the assay mix contained 20 nmoles of ATP to begin with this calibration curve deviates from the theoretical curve (dotted line Figure 6-2) by giving more incorporation than expected. Obviously, simply increasing the ATP concentration, while keeping the CTP, GTP and UTP concentrations constant causes an increase in incorporation of ^{3}H-ATP on salmon sperm DNA template. The effect of increasing the other NTP concentrations while holding the ATP concentration constant is given in Figure 6-3 and it shows that there is a stimulation of incorporation which reaches a limit. About 30% more incorporation is obtained if the GTP, CTP and UTP concentrations are raised from 0.1 mM to 1.2 mM while the ATP concentration is held at 0.4 mM.

Since the radioactive NTP in the cell extract would contain unknown amounts of the other 3 NTPs it was considered important to select assay conditions which buffered against fluctuations in incorporation on changing the NTP concentrations. It was therefore decided to increase the unlabelled NTP concentrations to 1.8 mM and using this new assay mix attempt to measure the amount and specific radioactivity of a known ^{32}P-ATP solution.

Figure 6-4 presents the calibration curve for an experiment of this type and comparison of this curve with that of Figure 6-2 shows that the stimulation of incorporation due to the addition of unlabelled ATP is greater when the ATP concentration is limiting as in Figure 6-4, however there is only a small difference between the two curves despite the large increase in the concentration of CTP, GTP and UTP from 0.1 mM to 1.8 mM.

In the experiment of Figure 6-4 the reduced incorporation corresponded to the addition of 19.5 nmoles of unlabelled ATP and therefore the ^{32}P-ATP specific radioactivity (3,250 cpm/pmole) is

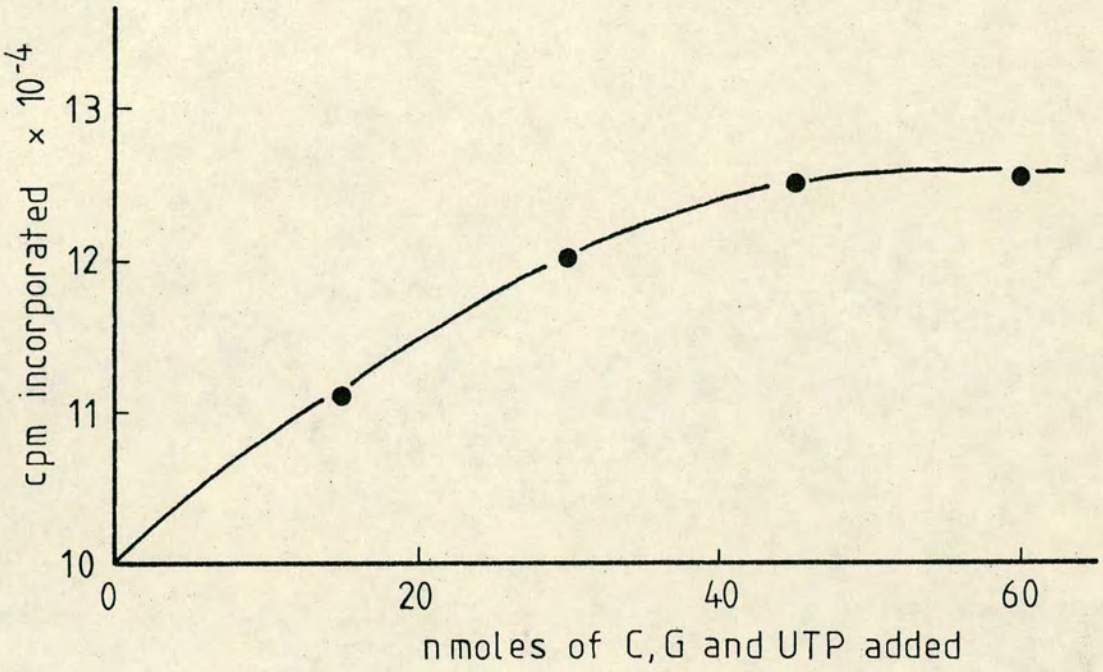


Figure 6-3 Effect of increasing the GTP, CTP and UTP concentrations at constant ATP concentration on the incorporation of ^{3}H-ATP into RNA by E. coli RNA polymerase

Using the same conditions as described in Figure 6-2 RNA polymerase assays were performed holding the ATP concentration at 0.4 mM (20 nmoles/50 μl) but adding either 0, 15, 30, 45 or 60 nmoles of each of GTP, CTP and UTP. Data is plotted as TCA precipitable cpm incorporated versus nmoles of NTP added.

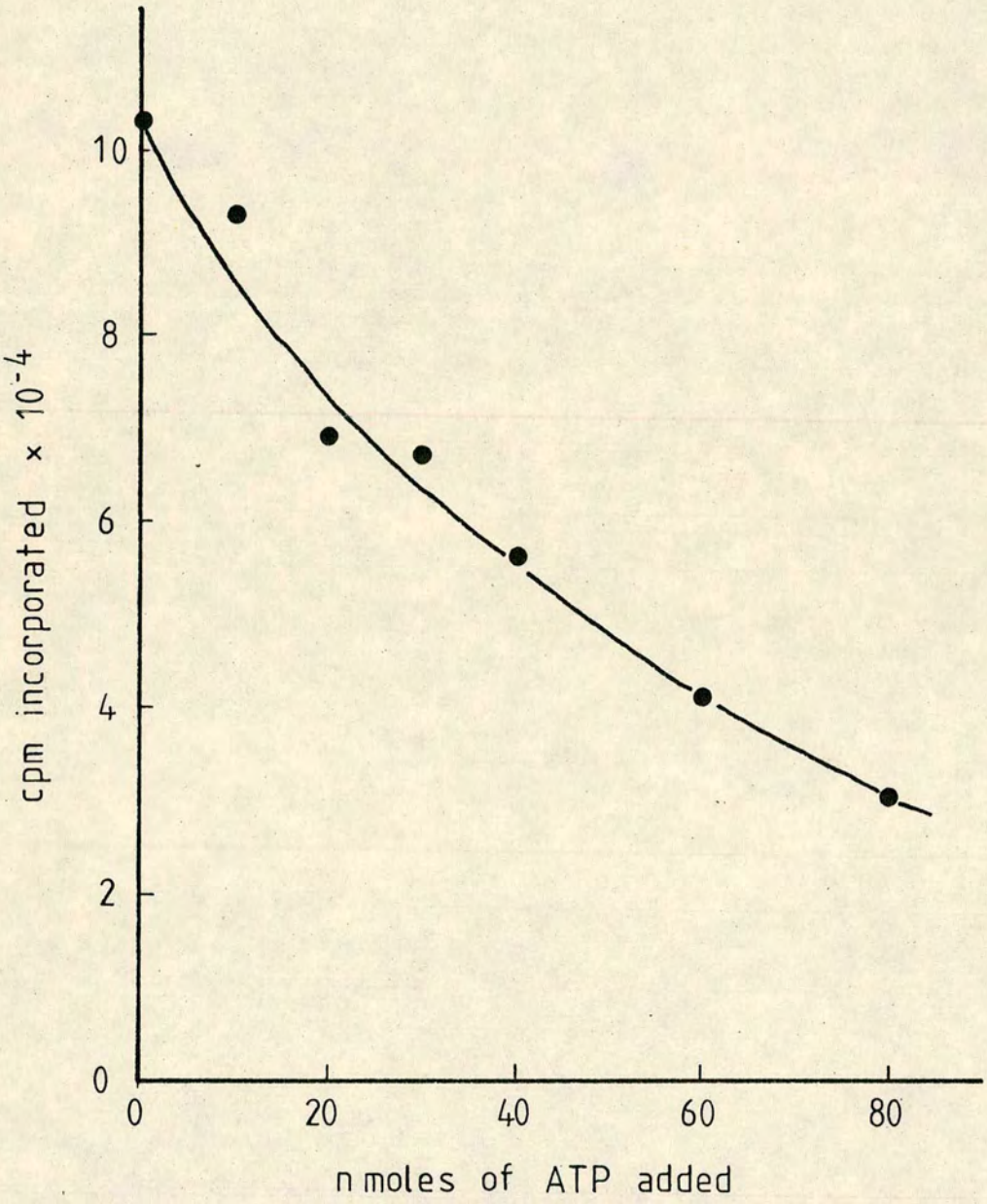

cpm incorporated × 10⁻⁴
10
8
6
4
2
0
0
20
40
60
80
n moles of ATP added

Figure 6-4 Measurement of specific radioactivity of ^{32}P-ATP standard solutions

Using the conditions detailed in method 2 (materials and methods section (o)) a calibration curve was constructed by adding various quantities of unlabelled ATP to the assay mix essentially as described in Figure 6-2, the data being expressed in the same way. Duplicate RNA polymerase assays were performed with and without the addition of 10 µl of two standard ^{32}P-ATP solutions each of 3,250 cpm/pmole, the first containing 16 nmoles of ATP per 10µl and the second 16 nmoles of ATP and 7.2 nmoles of each of GTP, CTP and UTP per 10 µl. The TCA precipitable cpm incorporated are given below together with the corresponding amounts of ATP added as read off the calibration curve and the values in brackets are averages. The specific radioactivities of the standard ^{32}P-ATP solutions were estimated in the following manner. Incorporation of 103,950 cpm ^{3}H-ATP (53,700 cpm/nmole) in the control sample corresponds to 1.94 nmoles of ATP incorporated. Therefore, assuming equal extents of reaction, 3,181 ^{32}P cpm also corresponds to 1.94 nmoles incorporated, which is a final specific activity of 1,640 cpm/nmole. If 19.5 nmoles of ATP have been added to 20 nmoles present, then the initial specific activity would have been 3,322 cpm/nmole (1,640 x 39.5 ÷ 19.5).

Volume of ^{32}P-ATP standard solutions (3,250 cpm/nmole) (µl)		^{3}H cpm incorporated	^{32}P - ^{3}H cpm incorporated	Amount of ATP added from curve (nmole)	Estimated specific activity (cpm/nmole)
	0	105,600			
	0	102,300			
		(103,950)			
- G, C and UTP	20	72,106	3,370		
	20	79,876	2,992		
		(76,000)	(3,181)	19.5	3,322
+ G, C and UTP	20	63,812	2,795		
	20	70,104	2,973		
		(67,100)	(2,884)	27.5	2,568

calculated to be 3,322 cpm/nmole which is in close agreement. The ^{32}P-ATP standard solution was also measured in the presence of 7.2 nmoles of each of CTP, GTP and UTP. In this case the ^{32}P-ATP specific radioactivity was measured as 2,568 cpm/nmole and the amount of ATP added as 27 nmoles. This result is the opposite of that expected since the addition of CTP, GTP and UTP even to the large amount present, ought to stimulate and not inhibit the incorporation. Consideration of the scatter of the points in the calibration curve and the duplicate measurements suggests that these determinations of the standard ^{32}P-ATP solution with and without added NTPs are essentially the same and it must be concluded that the combined error of the experiment is about 20-25%. This error was considered to be acceptable and it was therefore decided to measure the NTP content of a cell extract from stage 6 oocytes.

Figure 6-5 presents the results of an experiment of this type. Using the calibration curve the incorporation in the samples with PCA extract added correspond to ATP contents of about 75 nmoles in 20 oocytes, or about 4 nmoles per oocyte. This value is 8 times higher than that measured by Woodland & Pestell (1972) and this suggests that the PCA extract contains a factor which inhibits the RNA polymerase assay. This experiment highlights a weak point in this method of measuring NTP specific activity, since the determination depends critically on the extent of the polymerization reaction in the + and - samples being the same. Addition of a PCA extract from different sizes of oocytes could effect the polymerization in unknown ways and therefore the specific radioactivities may not even be accurate to within an order of magnitude. On the basis of this experiment it was decided to modify the approach.

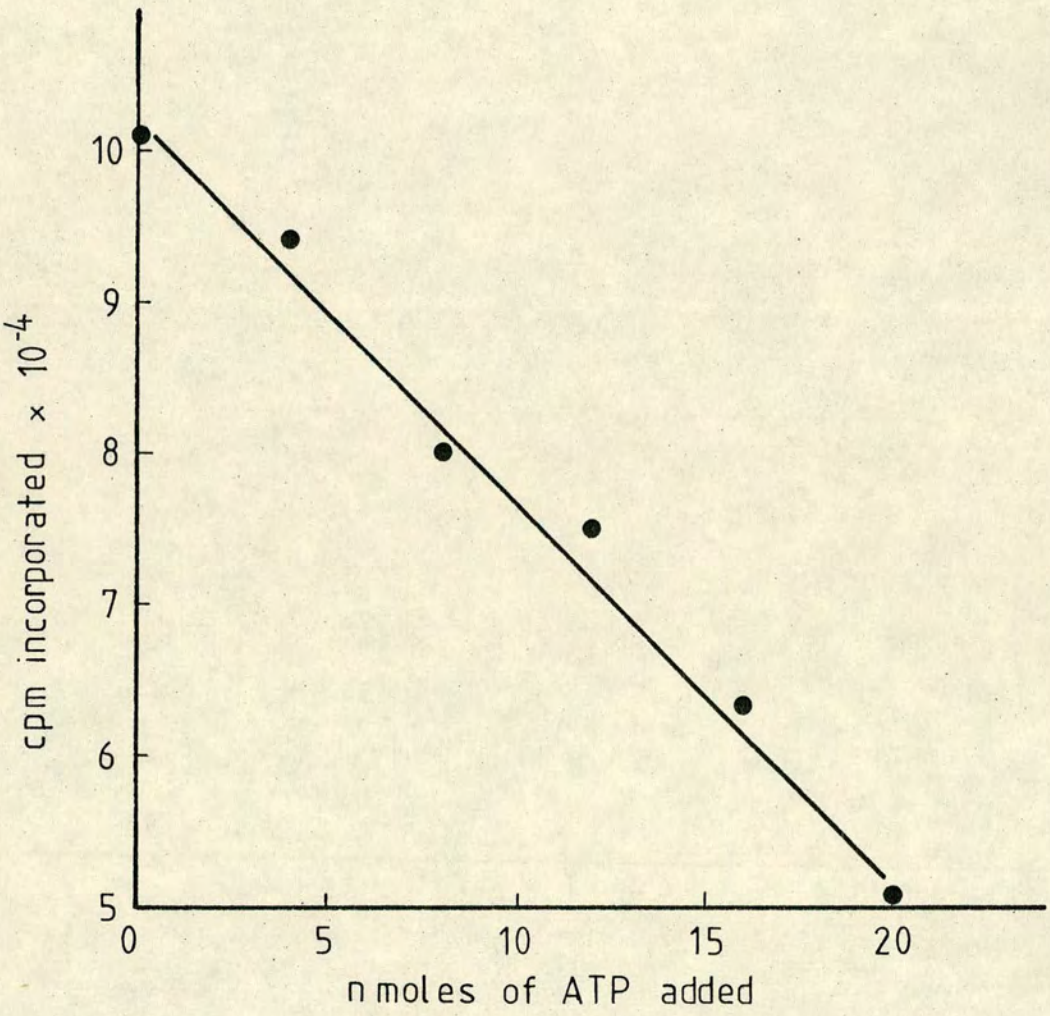

cpm incorporated × 10^{-4}
10
9
8
7
6
5
0
5
10
15
20
n moles of ATP added

Figure 6-5 Determination of the ATP pool size of stage 6 oocytes by an RNA polymerase assay

20 stage 6 oocytes were homogenized in 0.5 ml of ice cold 0.5 M PCA and the homogenate centrifuged at 1,000 g for 10 minutes at 4°C. The supernatant was neutralized by addition of a small volume of KOH using bromothymol blue as indicator, and then frozen at -20°C. After thawing, the salt was removed by centrifugation at 10,000 g for 10 minutes at 4°C. PCA extracts prepared in this manner could be concentrated by evaporation if necessary or used directly in RNA polymerase assays. Using the conditions outlined in method 2 (materials and methods section (o)) a calibration curve was constructed by adding known amounts of unlabelled ATP to RNA polymerase assays as in Figure 6-2. Aliquots of the PCA extract (above), which was redissolved in 40 µl, were added to control RNA polymerase assays and the ATP present determined by the reduction of ^{3}H-ATP incorporation into RNA. Data expressed as in Figure 6-2 and the values in brackets are averages.

Volume of PCA extract assayed (µl)	^{3}H cpm incorporated	Amount of ATP added from curve (nmoles)	Total ATP in PCA extract (nmoles)
10	54,700		
10	59,100		
	(56,000)	18.2	73
5	78,150		
5	76,050		
	(77,100)	9.9	79

New approach

Two alternatives were considered possible. The first alternative would eliminate the problem of different extents of reaction in the + and - samples by using the PCA extract itself as the source of NTPs for the assay mix. The addition of solutions of radioactive ATP, for example, of known specific radioactivities to a mixture of the PCA extract, DNA and enzyme would allow one to obtain an estimate of the ATP present in the extracts providing the stimulation of the incorporation due to adding the radioactive ATP solutions was not large. By comparing labelled and unlabelled PCA extracts the specific radioactivities of the NTP in the extract could be determined.

The second alternative was the method of Maxson & Wu (1976) which involved polymerizing two radio labelled NTPs, one of known specific radioactivity, into an alternating co-polymer using E. coli RNA polymerase and poly [d(A-T)] or poly [d(G-C)] as template. For example, to determine the ^{32}P-ATP specific activity in a PCA extract, ^{3}H-UTP of an appropriate known specific radioactivity would be added to the extract together with poly [d(A-T)] and enzyme. Since these two radio labelled NTPs must be incorporated in equimolar amounts the ratio of incorporation of the two isotopes into the RNA product equals the ratio of specific radioactivities of the two NTPs. If the ^{3}H-UTP specific activity was known then the ^{32}P-ATP specific activity would easily be calculated. It would be important to select a known specific radioactivity that would not be significantly diluted by its addition to the extract.

It is apparent that the extent of reaction is not important using this assay and no comparison between + and - samples is required. Therefore, in view of the fact that the RNA polymerase was a gift in

limited supply and, since the first alternative involved the selection of certain conditions where the stimulation of incorporation on addition of NTP was small, and since it would also involve obtaining sufficient data points to produce a reliable graph for extrapolation, the second assay method was selected. Following closely the method of Maxson & Wu, (1976), Table 6-1 details the results of an experiment to determine the specific radioactivity of a standard ^{32}P-ATP solution using ^{3}H-UTP of known specific radioactivity (325,800 cpm/nmole). The reverse experiment was also performed using a known ^{32}P-ATP solution (150,700 cpm/nmole) to measure ^{3}H-UTP specific radioactivity and also given is a measurement of the ^{3}H-UTP specific radioactivity used in experiment (A). All the results are in good agreement and in the three determinations the measured values do not differ from the known values by more than 25%.

These results were encouraging enough to attempt to measure the specific radioactivities of ^{3}H-UTP in stage 6 oocytes injected with ^{3}H-UTP and in previtellogenic ovary incubated in ^{3}H-uridine.

(d) Measurement of specific activity in stage 6 oocytes and previtellogenic ovary

These experiments were performed using the same conditions of microinjection and incubation as for similar experiments described in Chapters 4 and 5 for stage 6 oocytes and previtellogenic ovary respectively. At zero time and after 48 hours of incubation, batches of stage 6 oocytes which had been microinjected with ^{3}H-UTP were extracted with PCA and for previtellogenic ovary the times of PCA extraction were after 30 and 48 hours of incubation in ^{3}H-uridine. The neutralised extracts were then assayed with ^{32}P-ATP

Table 6-1 Determinations of NTP specific radioactivities

Sample A	Sample Volume	^{32}P - ^{3}H cpm	^{3}H cpm	Specific activity$_{3}$ determined using ^{3}H-UTP (325,800 cpm/nmole) cpm/nmole
^{32}P-ATP standard solution (2.3×10^6 cpm/nmole)	10µl	160,319	22,060	2.37×10^6
	10µl	172,540	25,100	2.24×10^6
	5µl	76,210	10,890	2.28×10^6
Sample B	**Sample Volume**	**^{32}P - ^{3}H cpm**	**^{3}H cpm**	**Specific activity$_{32}$ determined using ^{32}P-ATP (150,700 cpm/nmole) cpm/nmole**
^{3}H-UTP standard solution (1.1×10^6 cpm/nmole)	10 µl	77,440	693,423	1.35×10^6
	10 µl	71,040	601,520	1.28×10^6
	5 µl	54,761	427,174	1.18×10^6
^{3}H-UTP solution used in part A (0.326×10^6 cpm/nmole)	10 µl	58,925	122,680	0.314×10^6
	10 µl	54,318	119,482	0.331×10^6

Standard solutions of ^{32}P-ATP and ^{3}H-UTP were made up by adding known quantities of unlabelled ATP or UTP in aqueous solution to samples of ^{32}P-ATP or ^{3}H-UTP which had been evaporated to dryness in order to remove the ethanol present. The specific activities of these standard solutions were measured as described in Method 3 of materials and methods section (o). For the sample in (A) the specific radioactivity of the ^{32}P-ATP was found by multimplying the specific activity of the ^{3}H-UTP in the assay mix by the ratio of ^{32}P-^{3}H cpm: ^{3}H cpm. For the samples in (B) the ^{3}H-UTP specific activities were found by multiplying the specific activity of the ^{32}P-ATP in the assay mix by the ratio of ^{3}H cpm: ^{32}P - ^{3}H cpm.

(124,700 cpm/nmole) to determine the ^{3}H-UTP specific radioactivity as described above. Table 6-2 details the ^{3}H-UTP specific radioactivities obtained. For the injected stage 6 oocyte the zero time specific activity is about twice that measured after 48 hours. It must be pointed out that in this particular experiment the injected oocytes did not heal well and yolk leaked from them throughout the incubation.

The incorporation curve for this experiment is given in Figure 6-6A and it appears to differ from those presented in Chapter 4 in that it does not intercept close to the origin. However it is thought that this difference is probably due to the leakage of cytoplasm from the oocytes on the basis of the following reasoning. Since the injected ^{3}H-UTP swells the UTP pool by about 10% it is possible that the specific activity of ^{3}H-UTP remains constant until leakage has caused the UTP content to fall below some threshold value, after which time new synthesis of UTP occurs to re-establish the pool, and this would decrease the specific activity of the ^{3}H-UTP. If leakage continues the ^{3}H-UTP specific activity would continue to decrease. In this connection, it is interesting to note that the rate of incorporation over the first 17 hours is twice that measured using the 48 hour time point. If the zero time ^{3}H-UTP specific activity is used to calculate the amount of UTP incorporated over the first 17 hours and the 48 hour ^{3}H-UTP specific activity used to determine the amount in 48 hours, the same rate of incorporation is obtained (as shown in Figure 6-7A). It is therefore considered that the results for ^{3}H-UTP incorporation into total RNA in stage 6 oocytes given in Figure 6-7A are consistent with those presented in Chapter 4 for radioactive ATP and GTP incorporation.

Table 6-2 ^{3}H-UTP specific activities in microinjected stage 6 oocytes and in previtellogenic ovary incubated in ^{3}H-uridine

Sample	Sample Volume	^{32}P - ^{3}H cpm	^{3}H cpm	Specific activity determined using ^{32}P-ATP (124,700 cpm/nmole) cpm/nmole
Stage 6 oocyte Zero Time	10 µl	27,724	65,401	294,138
	5 µl	12,791	33,441	326,012
				(310,075)
Stage 6 oocyte 48 hr	10 µl	19,946	25,281	158,651
	5 µl	13,245	13,846	130,350
				(144,500)
PV ovary 30 hr	10 µl	34,885	28,786	102,888
	5 µl	21,275	17,667	103,540
				(103,214)
PV ovary 48 hr	10 µl	85,255	77,407	113,214
	5 µl	52,692	42,913	101,548
				(107,381)

Stage 6 oocytes were microinjected with 1 µCi ^{3}H-UTP each and incubated in batches exactly as described in Figure 4-1. At zero time and after 48 hours PCA extracts were prepared from the defolliculated oocytes as described in Figure 6-5 and RNA was extracted from the other batches of oocytes as in Figure 4-1. Previtellogenic ovaries were incubated in ^{3}H-uridine exactly as described in Figure 5-1 and RNA extracted at various times. After 30 and 48 hours PCA extracts were prepared from ovarian fragments and the ^{3}H-UTP specific activity of 5 and 10 µl samples from these and the stage 6 oocyte PCA extracts were determined using Method 3, section (o) of materials and methods, but the ^{32}P-ATP specific activity was 124,700 cpm/nmole. The ^{3}H-UTP specific activities were calculated exactly as described in Table 6-1 (B) and the average of the two determinations is given in brackets.

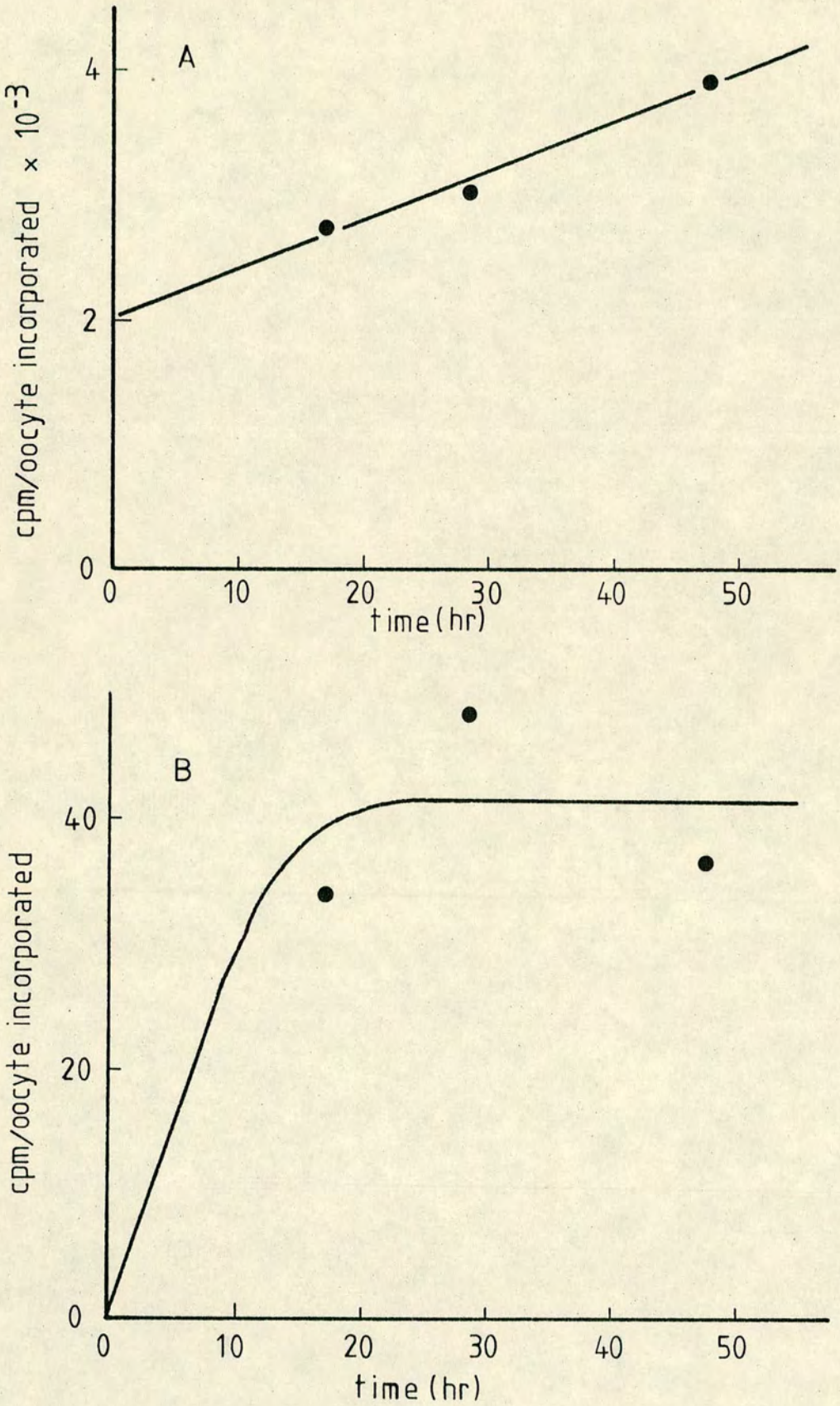

A
cpm/oocyte incorporated × 10⁻³
4
2
0
0
10
20
30
40
50
time (hr)
B
cpm/oocyte incorporated
40
20
0
0
10
20
30
40
50
time (hr)

Figure 6-6 Incorporation of ^{3}H-UTP into total RNA and oligo (dT) bound RNA by microinjected stage 6 oocytes

Samples of total RNA prepared from the stage 6 oocytes used in the experiment described in Table 6-2 were precipitated with TCA and the radioactivity determined as described in materials and methods section (m). Oligo (dT) bound RNA was prepared from the remainder of the total RNA as detailed in materials and methods section (h) and samples were precipitated with TCA as above. The recovery of loaded cpm after oligo (dT)-cellulose chromatography was over 96% and the figures show the cpm per oocyte incorporated into RNA as a function of time after microinjection.

(A) Incorporation into total RNA

(B) Incorporation into oligo (dT) bound RNA

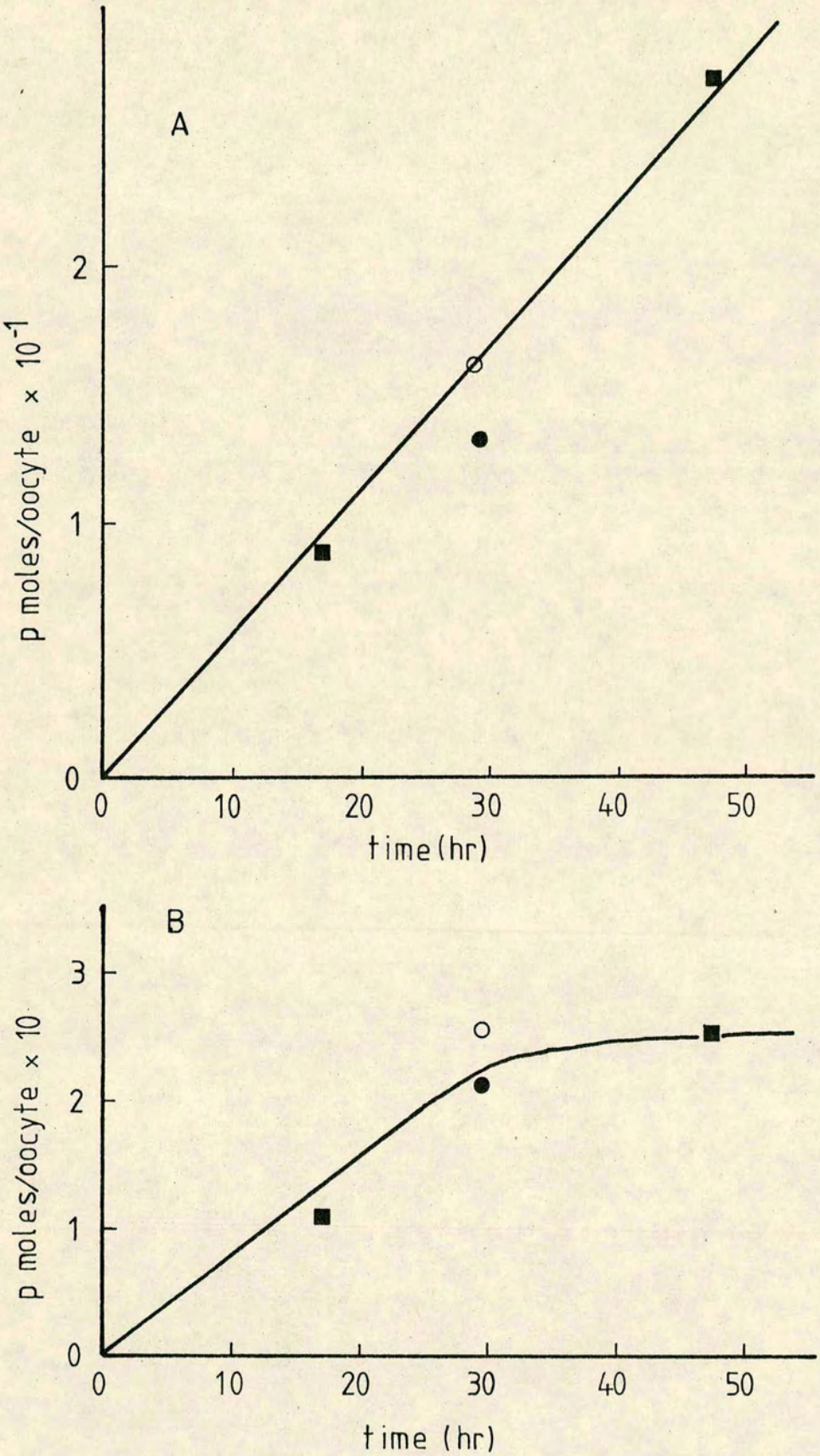

A
p moles/oocyte x 10^{-1}
0
1
2
0
10
20
30
40
50
time (hr)
B
p moles/oocyte x 10
0
1
2
3
0
10
20
30
40
50
time (hr)

Figure 6-7 Incorporation of ^{3}H-UTP into total RNA and oligo (dT) bound RNA in microinjected stage 6 oocytes

In the experiment described in Figure 6-6 and Table 6-2 the cpm/oocyte incorporated into both total RNA and oligo (dT) bound RNA were converted to pmoles/oocyte incorporated using the ^{3}H-UTP specific radioactivities determined in Table 6-2. For the 17 hour time points the zero time specific radioactivity (310 cpm/pmole) was used for the conversion and for the 48 hour time points, 145 cpm/pmole was used. For the 30 hour time point in figure 6-7B two values are given, the lower value (●) has been worked out using the average specific radioactivity of 277 cpm/pmole (marked (●) in Figure 6-7A) and the higher value (O) corresponds to a specific radioactivity of 190 cpm/pmole which is that required to place the 30 hour time point in Figure 6-7A on the straight line as shown.

(A) Incorporation into total RNA

(B) Incorporation into oligo (dT) bound RNA

Figure 6-6B also presents the data for incorporation of ^{3}H-UTP into oligo (dT) bound RNA by stage 6 oocytes. In terms of cpm/oocyte incorporated the results are consistent with the kinetics described in Chapter 4. In Figure 6-7B these results have been expressed as pmoles of ^{3}H-UTP incorporation/oocyte using the measured zero time and 48 hour specific activities. Taking these two points only, a slight saturation effect is observed. The position of the 30 hour time point cannot be determined with certainty since the specific radioactivity of the ^{3}H-UTP was not measured at this time point, however, two positions have been estimated and are shown in Figure 6-7B. The lower value was worked out using an average specific activity between 17 and 48 hours which would be consistent with a linear decrease in specific activity between 17 and 48 hours. The higher value was obtained by calculating the specific activity required to place the 30 hour time point for total RNA incorporation on the same straight line obtained using the 17 and 48 hour specific activities for total RNA. The specific radioactivity so obtained would require that there was a non linear decrease in specific radioactivity between 17 and 48 hours, the decrease being more rapid at first. Using either value reinforces the conclusion that incorporation of ^{3}H-UTP into oligo (dT) bound RNA by stage 6 oocytes saturates.

In Table 6-3 the actual values measured for ^{3}H-UTP specific activity in stage 6 oocytes are compared with the estimated value obtained using the method of Chapter 4. The estimated value is almost 4 times higher than the measured value. This suggests that the method used for estimating specific activities in injected oocytes in Chapter 4 tends to overestimate. The two most likely reasons for this difference are firstly, the assumption that all the

Table 6-3 Comparison of estimated and measured ^{3}H-UTP specific activity in microinjected stage 6 oocytes

	^{3}H-UTP specific activity (cpm/pmole)	Estimated / measured (zero time)
(A) Measured		
Using RNA polymerase assay (Table 6-2)	zero time 310.1 48 hours 144.5	
(B) Estimated		
^{3}H-UTP is 14 Ci/mmole		
1 μCi injected = 24 pmole		
The UTP pool size is 290 pmole/oocyte (Woodland & Pestell, 1972)		
Sum of cpm recovered = 355,800 cpm/oocyte		
Therefore, 355,800 ÷ (290 + 24) =	1,130	3.7
(C) Estimated		
Using a UTP pool size of 1,250 pmole/oocyte (Maller et al., 1977)		
355,800 ÷ (1,250 + 24) =	283	0.9

injected cpm are in the form of NTP. It is possible that during the drying down, redissolving and injection procedures some NTP is degraded. It is however unlikely that over 70% of the NTP should be destroyed in this way. The second reason is the value of 290 pmoles assumed for the pool size of UTP (Woodland & Pestell, 1972). If the UTP pool size was larger than 290 pmoles the estimated specific activity would be closer to the measured value. In this connection, a very recent publication (Maller et al. 1977) gives values for the NTP pool sizes in stage 6 Xenopus laevis oocytes which are 2-4 times greater than those of Woodland & Pestell (1972). Using the more recent value of 1,250 pmoles for the UTP pool size the estimated ^{3}H-UTP specific activity is in the range of measured values (Table 6-3). This calculation supports the view that the RNA polymerase assay for NTP specific activities is a valid procedure. Further, the rates of NTP incorporation by injected stage 6 oocytes given in Chapter 4 may all be underestimates since the smaller values for the NTP pool sizes were used. This point will be discussed later.

In Table 6-2 the measurements of the ^{3}H-UTP specific activity in previtellogenic ovary at the two time points are the same which supports the conclusion drawn in Chapter 5 that the specific activity in incubated previtellogenic ovary is constant from an early time.

Figure 6-8A gives the incorporation data for total RNA in previtellogenic ovary in terms of cpm/ μg RNA. ^{3}H-uridine incorporation into total RNA is linear in agreement with experiments in Chapter 5 and since the ^{3}H-UTP specific activity measured is constant, the incorporation data will remain linear if expressed as pmoles ^{3}H-UTP incorporated/ μg RNA. Figure 6-8B gives the kinetics of

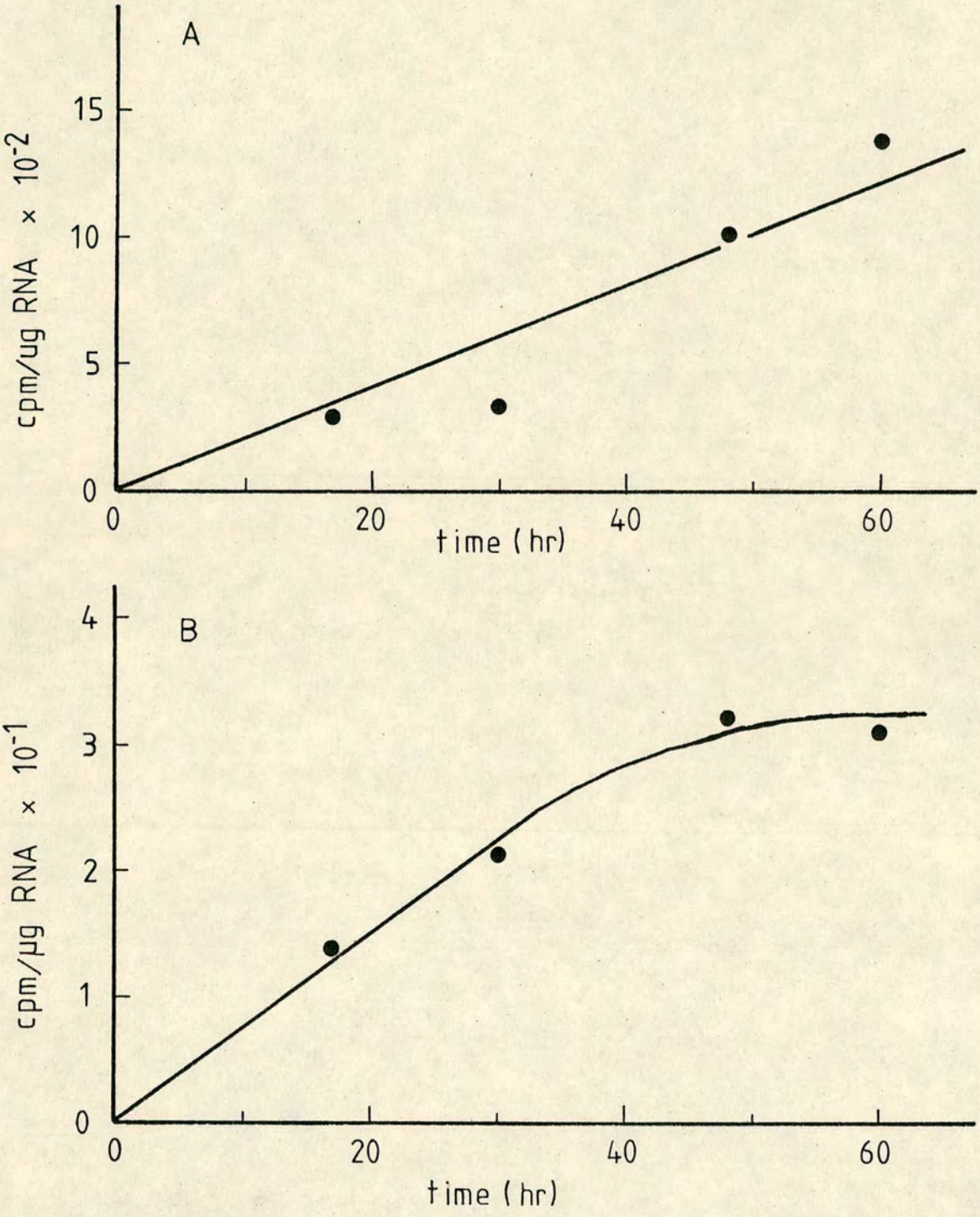

A
15
10
5
0
cpm/ug RNA × 10-2
0
20
40
60
time (hr)
B
4
3
2
1
0
cpm/µg RNA × 10-1
0
20
40
60
time (hr)

Figure 6-8 Incorporation of ^{3}H-uridine into total RNA and oligo (dT) bound RNA by previtellogenic ovary

Samples of total RNA prepared from the previtellogenic ovary used in the experiment described in Table 6-2 were precipitated with TCA and the radioactivity determined as described in materials and methods section (m). Oligo (dT) bound RNA was prepared from the remainder of the total RNA as described in materials and methods section (h) and samples were precipitated with TCA as above. The recovery of loaded cpm after oligo (dT)-cellulose chromatography was over 97% and the figures show the cpm of ^{3}H-uridine incorporated into RNA per μg of total RNA isolated, as a function of time.

(A) Incorporation into total RNA

(B) Incorporation into oligo (dT) bound RNA.

incorporation of ^{3}H-uridine into oligo (dT) bound RNA by previtellogenic ovary. The saturation curve observed is wholly consistent with those presented in Chapter 5.

Although in this experiment whole ovary was used both for the incorporation and the specific activity measurements, incorporation and rates of RNA synthesis per oocyte can be estimated if the following assumptions are made. Firstly, for the RNA content, the size of the oocytes were similar to those described in Figure 5-6 which had an average of 60 ng RNA/oocyte and therefore this value will be used in subsequent calculations. Secondly, one must assume that the ^{3}H-UTP specific activity measured for whole ovary is not significantly different from that of the previtellogenic oocytes themselves. This is probably true since when equilibrium has been established, even if the follicle cells have a higher ^{3}H-UTP specific activity their relative contribution to the average ^{3}H-UTP specific activity should be small due to their small size. Furthermore, measurements of the soluble cpm/ μg RNA for defollicled previtellogenic oocytes and previtellogenic ovary incubated in ^{3}H-uridine were very similar (Figure 5-6) and this does not suggest differential uptake of ^{3}H-uridine by previtellogenic oocytes and follicle cells. Accepting these assumptions for previtellogenic oocytes Table 6-4 compiles the rates of incorporation of ^{3}H into total RNA and oligo (dT) bound RNA for both stage 6 oocytes and previtellogenic oocytes. The significance of these rate measurements is discussed later.

Table 6-4 Comparison of rates of incorporation of ^{3}H into RNA in previtellogenic oocytes and stage 6 oocytes

Sample	A	B	C	D
	Specific activity from Table 6-2	Total RNA rate of incorporation of ^{3}H	Oligo (dT) bound RNA initial rate of incorporation	Oligo (dT) bound RNA steady state amount
	cpm/pmole	pmoles/oo/hr	pmoles/oo/hr	pmoles/oo
Previtellogenic oocyte	105.3	0.013 (16.5)	0.0005 (0.7)	0.018 (23)
Stage 6 oocyte	310.1	0.537 (670)	0.0066 (8)	0.25 (320)

Data compiled from the experiment described in Table 6-2 and Figures 6-7 and 6-8. For previtellogenic oocytes the rate of incorporation of ^{3}H-uridine into total RNA in Figure 6-8A in cpm/μg RNA/hour was converted to pmoles/oocyte/hour using the specific activity shown (column A) and an RNA content per oocyte of 0.06 μg. The initial rate and steady state amount of oligo (dT) bound RNA were calculated in a similar way using the data in Figure 6-8B. For stage 6 oocytes the values were taken directly from the curves in Figure 6-7A and B in which allowance has already been made for the difference in specific activity between zero time and 48 hours (Table 6-2). The numbers in brackets show the rates and amounts of RNA in pg/oocyte/hour or pg/oocyte calculated as detailed in materials and methods section (p).

(e) Discussion

In the experiments described in this Chapter the ability of E. coli RNA polymerase to synthesize RNA *in vitro* was exploited in attempts to measure the pool size and specific radioactivities of NTPs in *Xenopus laevis* oocytes.

The first method tried, involved setting up a standard curve relating the decrease in incorporation of a labelled NTP into RNA to the amount of the same unlabelled NTP added. This method was successful in measuring the specific radioactivities of certain standard solutions, but appeared to break down when measuring the actual pool size of NTP extracted from stage 6 oocytes. It appeared that some factor in the PCA extract inhibited the reaction and thus a very large pool size was obtained using the standard curve. The existence of any stimulatory or inhibitory factor is contrary to the observations of Sasvari-Szekely et al. (1975) and indeed there may not be an inhibitory factor present in the PCA extract since the value of the pool size taken from the literature to compare with the measured pool size may have been an underestimate.

Nevertheless the method was considered unsatisfactory and the recently published double-label method of Maxson & Wu (1976) was adopted. This method was rapidly successful and was used to measure the ^{3}H-UTP specific activities in previtellogenic ovary and stage 6 oocytes. For stage 6 oocytes the measured ^{3}H-UTP specific activity was nearly 4 times lower than had been estimated. This was considered to be due mainly to underestimating the UTP pool size, since using a larger recently published value for the UTP size gave similar estimated and measured specific activities. This observation then suggests that the rates of incorporation of ATP and GTP presented in Chapter 4 are also underestimates.

The values of ^{3}H-UTP specific activity measured for previtellogenic ovary are lower than for stage 6 oocytes and they support the conclusion that the NTP specific activity is constant from an early time in incubated ovary. It is argued that the average ^{3}H-UTP specific activity measured for previtellogenic ovary is not significantly different from that of the previtellogenic oocytes, and hence values for the rate of incorporation per oocyte are calculated. Rates of incorporation of ^{3}H-UTP into total RNA are about 40 times greater in stage 6 oocytes than in previtellogenic ovary, however incorporation into oligo (dT) bound RNA is only about 15 times greater. The significance of these results are discussed in the final Chapter.

CHAPTER 7

Discussion

(a) Introduction

Before discussing how the results obtained in this investigation relate to the present state of knowledge of RNA synthesis during oogenesis outlined in Chapter 1 it will be helpful to briefly review the main conclusions drawn in the preceeding chapters.

After a short lag, stage 6 oocytes accumulated stable RNA in the cytoplasm at a constant rate for about 100 hours and at least 80% of this RNA was 4S/5S RNA and rRNA, but some heterogeneous RNA may have been present. The initial rate of synthesis of the nuclear RNA was about $2\frac{1}{2}$ times greater than the rate of accumulation of cytoplasmic RNA and about 50% of the nuclear synthesis was due to heterogeneous RNA which had a half-life of less than 4 hours.

The kinetics of synthesis of poly(A)$^+$RNA levelled off after about 20-30 hours and no stable component was observed. Using radioactive GTP, in enucleatedoocytes, over 70% of the poly(A)$^+$RNA synthesis continued while total RNA synthesis was reduced by more than 80%. By preparing germinal vesicles most of the GTP labelled poly(A)$^+$RNA was in the cytoplasm at all times but using ATP about 40% of the label was in nuclear poly(A). The nuclear poly(A) isolated could be either free poly(A), a product of degradation of larger RNA or an artifact due to the nuclear isolation procedure but none of these alternatives were definitely excluded.

Similar kinetics of synthesis of RNA were observed in stage 1 oocytes but in this case 4S/5S RNA accounted for about 80% of the stable RNA synthesized although some rRNA may be synthesized by these oocytes. As in stage 6 oocytes, the incorporation curve for poly(A)$^+$RNA synthesis also levelled off within about 30 hours, but the sedimentation properties and poly(A) size of the newly synthesized

poly(A)$^+$RNA in stage 1 oocytes were slightly different from those of stage 6 oocytes. In a number of different experiments very similar rates of incorporation of radioactive nucleoside by stage 1 oocytes were observed.

Finally in Chapter 6 the actual specific activity of the precursor NTP pool was determined for both oocyte stages and it was concluded that the estimates of the rates of RNA synthesis in stage 6 oocytes made in Chapter 4 were about 3 fold too low.

(b) Ribosomal RNA

As outlined in Chapter 1 previous estimates for the rate of rRNA (stable RNA) synthesis in stage 6 Xenopus laevis oocytes cover a wide range 0.6-4.2 ng/oocyte/hour, and the variability has been put down to differences in the physiological status of the females used which to some extent may be seasonal (Scheer, 1973; LaMarca et al., 1973, 1975; Anderson & Smith, 1977). Estimates of the stable rate of RNA synthesis determined in this investigation are compiled in Table 4-3 and have been derived using assumptions outlined in Chapter 4 to generate values for the precursor specific activities. These values in Table 4-3, which are minimum estimates, average about 0.2 ng/oocyte/hour. For reasons discussed in Chapter 6 a correction of about 3 fold should be applied to these values, and this correction would give a new average minimum estimate of the rate of stable RNA synthesis in vitro of 0.6 ng/oocyte/hour which agrees quite well with that in Table 6-4 and those of other workers (0.6-2.0 ng/oocyte/hour) who studied RNA synthesis in vitro (LaMarca et al., 1973, 1975; Anderson & Smith, 1977) but all these values are at least 2 fold lower than the in vivo measurement of Scheer (1973).

The third explanation offered by Colman (1974) was that of atresia of large stage 6 oocytes and he reported a value of 2% atretic oocytes in his studies. However, there are a minimum of 10,000 oocytes in the ovary of a mature female (Ford, personal communication) and 13% or more of these will be stage 6 oocytes (Dumont, 1972). If this population of stage 6 oocytes must be replaced in about 1 month a minimum of 4% per day must be resorbed. Colman's value of 2%, which is greater than observations in this laboratory, suggests that atresia at this rate could possibly account for the observation of a constant RNA content in stage 6 oocytes providing the actual process of resorption did not take much longer than 1 day, otherwise the percentage of stage 6 oocytes which were being resorbed should be greater than that observed. This requirement makes the explanation of atresia somewhat unlikely.

Consequently the remaining explanation for the constant RNA content of stage 6 oocytes is that of RNA turnover. If, as suggested by Anderson & Smith (1977), the 4 μg rRNA in the stage 6 oocyte is a steady state amount maintained by a synthetic rate of 0.6 ng/oocyte/hour then half of the rRNA should turnover every 190 days which seems inconsistent with the idea that ribosomes are being stored for use in embryogenesis. Furthermore, the observations that over 90% of other classes of RNA synthesised early in oogenesis are stable for at least 560 days (Ford et al., 1977) and that the stability of mRNA microinjected into stage 6 oocytes is very great (Gurdon, 1974), suggest that in the cytoplasm of oocytes a mechanism exists which stabilizes a range of RNA molecules. Thus either rRNA is not subject to this stabilization or the idea that the whole rRNA population turns over is incorrect.

An alternative explanation for the constant RNA content in stage 6 oocytes in the face of continued synthesis could be that there is a factor limiting the total number of ribosomes to about 10^{12} per oocyte. Then, if all the newly synthesized 18S and 28S RNA turned over with the half-life of about 20 days measured in vivo (Leonard & LaMarca, 1975) which is sufficiently long not to have been noticed in Figure 4-2, a steady state amount of 0.4 μg of rRNA would result. While this quantity of RNA is equivalent to about 10% of the total number of oocyte ribosomes and only about 1% of the total number of ribosomes are present in polysomes (Rosbash & Ford, 1974; Woodland, 1974), it is possible that some of the newly synthesized 18S and 28S RNA is never assembled into ribosomes. Even assuming that a third of the low molecular weight RNA synthesized in stage 6 oocytes (Table 7-1) is 5S RNA and in several studies synthesis of stable 5S RNA in this quantity has not been observed (LaMarca et al., 1973; Colman, 1974; Anderson & Smith, 1977), then 18S and 28S RNA molecules are synthesized in excess of 5S RNA molecules. If insufficient 5S RNA molecules or even ribosomal proteins are available, either from storage or synthesis, to permit the assembly of all of the newly synthesized 18S and 28S RNA into intact ribosomes, it is possible that the excess 18S and 28S RNA could turn over.

However, even if the real significance of rRNA synthesis in stage 6 Xenopus oocytes is not clear, it is apparent that during oogenesis a massive activation of rRNA synthesis occurs. The rate of stable RNA synthesis in stage 1 Xenopus oocytes was measured at about 16 pg/oocyte/hour (Table 6-4). Since these oocytes contained about 60 ng RNA and must have been a maximum of 6 months old, an average rate of synthesis of stable RNA over this period would be 14 pg/oocyte/hour and on this basis it would seem that there is a constant rate of

Table 7-1 Comparison of rates of RNA synthesis in stage 1 and stage 6 oocytes of Xenopus laevis

Class of RNA	Rate of RNA synthesis			Approx. gene number	Rate of tran- scriptn per gene n/s	Polymerase packing on gene	Tran- scription rate per polymerase n/s
	pg/oo/hr	n/s	molec./s				
Stage 6 oocytes							
rRNA *in vitro*	600	3.1×10^{8}	4.8×10^{4}	2×10^{6}	155	100	2
rRNA *in vivo*	4,200	2.1×10^{9}	3.2×10^{5}	2×10^{6}	1,050	100	11
4S RNA	20	1×10^{7}	1.4×10^{5}	4×10^{4}	250	1-2	125-250
5S RNA	<10	5×10^{6}	4.2×10^{4}	5×10^{4}	100	1-2	50-100
poly(A)$^{+}$RNA	30	1.6×10^{7}	8×10^{3}	1.3×10^{4}	1,230	25	50
(if up to 30% is nuclear)	<9	$<4.8\times10^{6}$	$<2.4\times10^{3}$	1.3×10^{4}	<370	25	<15
hnRNA	1500	7.8×10^{8}	-	-	-	-	-
Stage 1 oocytes							
rRNA *in vitro*	<1	5×10^{5}	77	2×10^{6}	0.25	100	0.0025
4S RNA	5	2.6×10^{6}	3.7×10^{4}	4×10^{4}	65	1-2	32-65
5S RNA	8	4.2×10^{6}	3.5×10^{4}	5×10^{4}	84	1-2	40-80
poly(A)$^{+}$RNA	0.7	3.6×10^{2}	1.8×10^{2}	1.3×10^{4}	27	25	1
hnRNA	13	6.7×10^{6}	-	-	-	-	-

Data compiled from experiments in Chapter 4 and 5 for stage 6 and stage 1 oocytes respectively or as calculated in the text. Rates in pg/oocyte/hour were converted to nucleotides/second (n/s) using an average molecular weight of 321 daltons for the nucleotides incorporated and rates in n/s were converted to molecules/second using 6,500; 70; 120 and 2,000 for the number of bases per molecule for rRNA, 4S RNA, 5S RNA and poly(A)$^{+}$RNA respectively. Gene numbers are taken from the sources detailed in Chapter 1 and the polymerase packing of about 100 per ribosomal gene taken from Miller & Beatty (1969b). A similar packing ratio has been assumed for the other classes of RNA.

stable RNA synthesis during previtellogenesis and that, as was found for stage 6 oocytes, *in vivo* and *in vitro* rates are comparable. Thus, stable RNA synthesis in stage 6 oocytes is about 40 fold higher than in stage 1 oocytes.

In Figure 5-7 the proportion of stable RNA synthesis in stage 1 oocytes due to rRNA was about 6%, and about 80% was contributed by 4S/5S RNA. These values are a little higher than previous estimates in which heterogeneous RNA accounted for a greater proportion of the labelled RNA (Thomas, 1974). 4S and 5S RNAs are synthesized in roughly equal amounts in stage 1 oocytes (Thomas, 1974; Ford et al., 1977) and on this basis Table 7-1 presents the rates of various RNA classes in stage 1 and also in stage 6 oocytes. Since similar rates of stable RNA synthesis have been observed in stage 3 and stage 6 oocytes (LaMarca et al., 1973), the rate of rRNA synthesis increases at least 600 fold at about vitellogenesis and the activation must persist longer than the lampbrush chromosome stage. This situation is somewhat different from that observed in the newt *Triton alpestris* (Scheer et al., 1976) where a maximum rate of rRNA synthesis occurs during midvitellogenesis. In this urodele the rate of RNA synthesis in previtellogenic oocytes (stage 1 equivalent) is 0.01-0.5% of the mid-vitellogenic rate and the full grown oocyte rate is about 13% of the mid-vitellogenic rate. In *Triton* these rate changes have been correlated with differences in RNA polymerase packing on the nucleolar ribosomal genes (Scheer et al., 1976) and it is likely that differences in packing ratio are applicable in *Xenopus* (Anderson & Smith, 1977) but if so the packing ratio in stage 3 and stage 6 oocytes should be similar. At a rate of stable RNA synthesis of 16 pg/oocyte/hour stage 1 oocytes would accumulate 0.14 μg RNA in 1 year which is approximately the RNA content of a

stage 3 oocyte (Scheer, 1973; Rosbash & Ford, 1974; Table 1-1) and in a further 9 months synthesis of stable RNA at a rate of 0.6 ng/oocyte/hour would result in about 4 μg RNA being present. These *in vitro* measurements are commensurate with the length of oogenesis in *Xenopus laevis*.

(c) 5S RNA

In stage 6 oocytes of *Xenopus laevis*, synthesis of 4S/5S RNA accounts for about 5% of the stable RNA synthesis and not more than about one third of this low molecular weight RNA has been attributed to 5S RNA (LaMarca et al., 1973; Colman, 1974; Anderson & Smith, 1977; Brown & Gurdon, 1977). Consequently the rates given in Table 7-1 show that the rates of synthesis of 5S RNA in stage 1 and stage 6 oocytes are similar and since similar patterns of newly synthesized RNA were observed in stage 3 and stage 6 oocytes (LaMarca et al., 1973), this would suggest a roughly constant 5S RNA synthetic rate throughout oogenesis. Certainly an increase of more than 5 fold can be ruled out. In Table 7-1 it is seen that in stage 1 oocytes 5S RNA molecules are synthesized about 470 fold faster than 18S and 28S RNA molecules and therefore the activation of 18S and 28S RNA synthesis of 600 fold, with little change in the 5S RNA rate, makes the molar rates of synthesis of these 3 RNA species approximately equal. This value for the molar excess of 5S RNA synthesis over 18S and 28S RNA synthesis is considerably greater than that of Ford (1972) who obtained a maximum value of 20 fold molar excess. Excluding heterogeneous RNA, 18S and 28S RNA would have to account for about 40% of the newly synthesized stable RNA in stage 1 oocytes to obtain this molar excess. In Figure 5-3 this value is not reached for ovary RNA

where most of the incorporation into 18S and 28S RNA is due to follicle cells (Thomas, 1974; Chapter 5), therefore, despite differential homogenization significant follicle cell RNA must have contaminated the oocyte cell fractions in the earlier work (Ford, 1972).

(d) 4S RNA

The pattern of synthesis of 4S RNA during oogenesis is similar to that just described for 5S RNA. Even if all the low molecular weight RNA synthesis in stage 6 oocytes was due to 4S RNA synthesis a maximum activation of about 6 fold could occur during oogenesis. Thus the rate of 4S RNA synthesis must also be roughly constant throughout oogenesis and its similarity to the rate of 5S RNA synthesis means that approximately equal amounts of 4S and 5S RNA will have accumulated by the end of oogenesis (Ford, 1971; Rosbash & Ford, 1974).

(e) Poly(A)$^+$RNA

From Table 6-4 the initial rate of synthesis of poly(A)$^+$RNA in previtellogenic oocytes is about 0.7 pg/oocyte/hour. At this rate the time required to synthesize the 40 ng poly(A)$^+$RNA present in stage 2-6 oocytes (Rosbash & Ford, 1974) would be about $6\frac{1}{2}$ years. Since the 40 ng is accumulated by stage 2 and takes about 1 year, an average rate of synthesis over this period would be nearer 5 pg/oocyte/hour. Further, since the poly(A)$^+$RNA made *in vitro* in stage 1 oocytes appears to be unstable, yet poly(A)$^+$RNA made *in vivo* in these oocytes is very stable (Ford et al., 1977), it must be concluded that *in vivo* rates of poly(A)$^+$RNA synthesis at least 10 fold

in excess of those measured in vitro occur at some time during previtellogenesis and that either the unstable poly(A)$^+$RNA synthesized in vitro is a different class of poly(A)$^+$RNA from that stored, or that in vivo the oocyte has a means of selecting certain poly(A)$^+$RNA molecules for stabilization and storage.

Although arguments have been presented which suggested that in both stage 1 and stage 6 oocytes incubated in vitro the kinetics of synthesis of 4S, 5S and 18S and 28S RNA are comparable with in vivo measurements both qualitatively and quantitatively, it is known that mRNA synthesis is responsive to hormone action in other systems (O'Malley et al., 1969; Mueller et al., 1971; Means et al., 1972) and therefore the kinetic pattern of poly(A)$^+$RNA synthesis observed in vitro in stage 1 oocytes may be a basal rate of synthesis of unstable RNA or a stable rate of synthesis declining as some factor (e.g. hormone) produced in vivo becomes limiting in vitro. Since even the initial rate of poly(A)$^+$RNA synthesis in vitro in stage 1 oocytes is too low to account for the amount of maternal mRNA stored, then if in vitro incubation is responsible for a change in poly(A)$^+$RNA synthesis it must cause both a drop in the initial rate of synthesis and a decrease in stability of the RNA class. However, this cannot be the case for stage 6 oocytes since similar kinetics of poly(A)$^+$RNA synthesis are observed using oocytes 0, 1 and 4 days after removal from the animal.

This paradox, that no stable poly(A)$^+$RNA is synthesized in vitro during the stage of oogenesis when maternal mRNA appears to accumulate (Rosbash & Ford, 1974), could be resolved if the maternal mRNA is initially synthesized with little or no 3' poly(A) and not polyadenylated until after 2-3 days or longer. If this were the case the

poly(A)$^+$RNA synthesized in stage 1 oocytes should be a different class to that stored and also stable heterogeneous RNA should be present in these oocytes. In this connection Thomas (1974) observed significant amounts of heterogeneous RNA in the cytoplasm of stage 1 oocytes of Xenopus (see next section).

In experiments in Chapter 5 which compared the length of the 3' poly(A) sequence of newly synthesized and steady state molecules in stage 1 oocytes, two thirds of the radioactivity came from time points after the steady state had been reached yet only one third of the poly(A) sequences were within the steady state size distribution. This observation, if significant, suggests that stored poly(A)$^+$RNA molecules in stage 1 oocytes have slightly shorter poly(A) tails or in vitro the poly(A) processing is not identical to that which occurs in vivo.

The amount of newly synthesized poly(A)$^+$RNA accumulated in vitro at the steady state is 23 pg/oocyte (2×10^7 molecules of 2,000 bases) which is only 0.06% of the stage 2-6 amount (40 ng) (Rosbash & Ford, 1974) and this quantity of RNA could easily be accommodated in polysomes (Rosbash & Ford, 1974; Darnbrough & Ford, 1976). The instability of this class of RNA if it were being translated could account for subsequent changes in the pattern of protein synthesis during oogenesis

From Table 4-3 the average initial rate of synthesis of poly(A)$^+$RNA in intact stage 6 oocytes is about 30 pg/oocyte/hour (10 pg/oocyte/hour x 3 fold correction factor) and although synthesis of poly(A)$^+$RNA at this rate would allow accumulation of 40 ng in about 2 months, the kinetic data shows that all this RNA is unstable and only 0.5% of 40 ng is accumulated at the steady state. Experiments in

Chapter 4 showed that the stage 6 oocyte poly(A)$^+$RNA synthesis was largely insensitive to enucleation but very sensitive to the drug ethidium bromide which strongly suggested that much of the poly(A)$^+$RNA synthesis was mitochondrial in origin. This interpretation seems more probable in the light of other studies. The minimum rate of synthesis of apparently stable mitochondrial total RNA in Xenopus laevis has been measured at 21-45 pg/oocyte/hour and some 30% of the radioactive RNA was thought to be polyadenylated (Webb et al., 1975). Clearly the average rate measured above is within this range and the steady state amount made would correspond to about 3 molecules of poly(A)$^+$RNA per mitochondrial genome (500 pg poly(A)$^+$RNA = 5 x 10^8 molecules of 2,000 bases ÷ 1.8 x 10^8 mitochondrial genomes = 3). Since the mitochondrial genome can code for about 8 proteins (Perlman et al., 1973; Hirsch et al., 1974; Devlin, 1976) this suggests that at the steady state few mitochondria are active in synthesizing poly(A)$^+$RNA in stage 6 oocytes. In addition, the rate of mitochondrial rRNA synthesis of 13 pg/oocyte/hour (Webb et al., 1975) is equivalent to 3% of the mitochondria synthesizing only one rRNA precursor per hour. It must be concluded that stage 6 oocyte mitochondria are relatively inactive in RNA synthesis although it may be that during the period of intense mitochondriogenesis in stage 2/3 oocytes (Webb & Smith, 1977) that mitochondrial RNA synthesis is greater. Comparison of the rates of poly(A)$^+$RNA synthesis in stage 1 and stage 6 oocytes in Table 7-1 shows that in vitro the stage 1 rate is about 40 fold lower than in stage 6 oocytes. Since the mitochondrial DNA content is only 5-10 fold lower in stage 1 oocytes (Webb & Smith, 1977), then even if all of the poly(A)$^+$RNA synthesis in stage 1 oocytes was mitochondrial, it must be that mitochondria are less active in poly(A)$^+$RNA synthesis in vitro in stage 1 oocytes

than in stage 6 oocytes. This conclusion is even more valid if little of the poly(A)$^+$RNA synthesis in stage 1 oocytes is mitochondrial and certainly in Chapter 5 the evidence suggested that stage 1 and stage 6 oocyte newly synthesized poly(A)$^+$RNA were different classes of RNA.

In a recent review Davidson (1976) reported unpublished data of Dolecki & Smith on the rate of poly(A)$^+$RNA synthesis in Xenopus laevis oocytes. Values for stage 3 oocytes were 1.5-2 x less than stage 6 oocytes but all values were 5-10 fold higher than the average value reported here for stage 6 oocytes. Since no mention was made of the instability of this class of RNA in stage 6 oocytes it is likely that initial rates were measured over a short time course and this may partly account for the higher values obtained by these workers, and indeed the highest values in Table 4-3 are close to the values reported in Davidson's review. If much of the poly(A)$^+$RNA synthesis in stage 3 as well as stage 6 oocytes is due to mitochondria then the $1\frac{1}{2}$-2 fold lower rate observed in stage 3 oocytes could be accounted for by the fact that there is about 1.4-2.2 fold less mtDNA in stage 3 oocytes (Webb & Smith, 1977).

Although in vitro the poly(A)$^+$RNA synthesis in stage 6 oocytes has been considered to be largely due to mitochondria, up to about 30% or even more, as a result of the indirect methods of analysis used, of this poly(A)$^+$RNA synthesis could be nuclear in origin. Either, any non-mitochondrial fraction of the newly synthesized poly(A)$^+$RNA in stage 6 oocytes represents a different class of poly(A)$^+$RNA from that stored or the oocyte has a mechanism for keeping part of a class of mRNA molecules very stable while another part of the same class turns over. The stability of the poly(A)$^+$RNA

may be acquired during early oogenesis perhaps by complexing with certain proteins and the same molecules synthesized later in oogenesis may be unstable due to the absence of these proteins. The in vivo pattern of protein synthesis in stage 1 and stage 6 oocytes are quite different (Darnbrough & Ford, 1976).

In either case the poly(A)$^+$RNA synthesized in vitro may represent mRNA molecules which are being translated in stage 6 oocytes. In this connection, the steady state amount of newly synthesized poly(A)$^+$RNA of 0.5 ng is less than the 1 ng of mRNA in polysomes in stage 6 oocytes (Woodland, 1974), and if the newly synthesized poly(A)$^+$RNA in stage 1 oocytes is also mRNA being translated then the differences observed in these two classes of poly(A)$^+$RNA might account for the change in protein synthesis during oogenesis. If the stage 3 oocyte synthesizes non-mitochondrial poly(A)$^+$RNA at a similar rate to the stage 6 oocyte then the activation in poly(A)$^+$RNA synthesis (Table 7-1) will have occured at about the same stage as the activation of 18S and 28S RNA, and will have persisted longer than the lampbrush chromosome phase.

(f) HnRNA

In Chapter 4 synthesis of heterogeneous RNA was observed in the germinal vesicles of stage 6 oocytes, and the properties of this hnRNA were similar to those reported recently (Anderson & Smith, 1977). On sucrose gradients, much of this RNA sedimented in the size range 4-40S and little radioactivity was present in >40S fractions. According to the data of Anderson & Smith, (1977) for Xenopus laevis approximately 6% of the hnRNA was >40S in size in steady

state conditions, and the initial rates of synthesis of >40S and 4-40S heterogeneous RNA amounted to about 1.5 ng/oocyte/hour. Similar values were obtained in this investigation (Table 4-1).

Since, comparable initial rates of hnRNA synthesis in vitro are reported to occur in stage 3 Xenopus oocytes (Anderson & Smith, 1977), and the rate of poly(A)$^+$RNA synthesis in these two oocyte stages are believed to be similar (Davidson, 1976), it is likely that in Xenopus observations on the poly(A) content of hnRNA in stage 6 oocytes may in general apply to lampbrush chromosome stage oocytes. In Table 4-5 approximately 7.5% of the total incorporation of ATP into RNA in the germinal vesicle was into poly(A). Assuming a base composition for the nuclear RNA of approximately 25% adenylic acid the percentage poly(A) in total nuclear RNA would be about 2%. Since in steady state conditions roughly half of the nuclear RNA is rRNA precursor (Anderson & Smith, 1977; Figure 4-7) the percentage poly(A) in hnRNA is approximately 4% in Xenopus laevis. However, in Chapter 4 the significance of this nuclear poly(A) was not clear and the most likely explanation provided was that of abnormal poly(A) metabolism occuring during nuclear isolation. As a result of recent work on Rana pipiens (Rogers & Browder, 1977b), this conclusion may be less likely. By differential nuclease digestion these workers obtained a minimum estimate of about 1.5% poly(A) in nucleoplasmic RNA in intact lampbrush stage oocytes, but this technique does not distinguish between free poly(A) and poly(A) covalently linked to hnRNA. While it is possible that observations made using a different species may not apply to stage 6 Xenopus oocytes it is worth reconsidering the arguments of Chapter 4. If free poly(A) is not produced during the manipulative procedures then it must exist in the germinal vesicle. If the poly(A) is

covalently linked to hnRNA molecules one would expect much more radioactivity binding to oligo (dT)-cellulose in intact oocytes than in manipulated ones, since in manipulated oocytes these putative poly(A)$^+$hnRNA molecules appear to be degraded, unless intact poly(A)$^+$hnRNA molecules fail to bind to oligo (dT)-cellulose or unless only the poly(A) is labelled and the rest of the hnRNA is not labelled. The alternative explanation is that the poly(A) exists as free poly(A) in the nucleus.

It appears that in amphibia some classes of hnRNA have a high uridylic acid content (Sommerville, 1973; Anderson & Smith, 1977) and if this is due to oligo (U) tracts as have been reported in hnRNA in Hela cells (Edmonds et al., 1976), then it is possible that newly labelled poly(A)$^+$hnRNA may not bind to oligo (dT)-cellulose because the poly(A) is hybridized to oligo (U). If this were the case the poly(A):oligo (U) hybrid should be resistant to RNAse degradation, but since the RNA is melted and chilled before oligo (dT)-cellulose chromatography the poly(A) alone could be recovered. RNAse resistant fragments of hnRNA have been reported (Sommerville & Malcolm, 1976).

If the newly labelled poly(A) was added to unlabelled hnRNA, this would suggest the existence of very stable hnRNA and unusual hnRNA processing in Xenopus oocytes. Finally, if poly(A) was free in the nucleoplasm, while it should have been detected in the experiments in Chapter 4, it might suggest that no poly(A)$^+$mRNA enters the cytoplasm due to intra nuclear removal of the poly(A). This would be in agreement with the observations that no poly(A)$^+$RNA accumulates in the stage 6 oocyte (Rosbash & Ford, 1974) and that most of the poly(A)$^+$RNA synthesized in stage 6 oocytes appears to be mitochondrial (Chapter 4).

No information about the presence of poly(A) in the nuclei of stage 1 Xenopus oocytes is available, and in fact the synthesis of hnRNA is poorly characterized. However, from the work in this investigation on the rate of RNA synthesis in stage 1 oocytes (Chapter 5) and earlier work in which stage 1 germinal vesicles were prepared (Thomas, 1974) it is possible to estimate the rate of synthesis of hnRNA. After 24 hours of labelling about 25% of the total incorporation was into > 5S heterogeneous RNA in the germinal vesicle of stage 1 Xenopus oocytes (Thomas, 1974).

In Table 6-4 the rate of total RNA synthesis in stage 1 oocytes was estimated to be about 16 pg/oocyte/hour. Therefore after 24 hours 25% of the RNA synthesized will amount to 98 pg of hnRNA. If this is a steady state amount accumulated with a half-life of about 5 hours (the half-life reported for most of the stage 6 oocyte hnRNA) the initial rate of synthesis would be 13.5 pg/oocyte/hour and this value has been entered in Table 7-1. Comparison with the rate of hnRNA synthesis in stage 6 oocytes shows an activation of about 100 fold, which is of the same order as the apparent activation of poly(A)$^+$RNA synthesis but lower than the rRNA activation. Thus, although the activation of poly(A)$^+$RNA may not be real if it is mainly due to mitochondria, there is a clear activation of hnRNA synthesis at about vitellogenesis. Since no accumulation of poly(A)$^+$RNA occurs after vitellogenesis (Rosbash & Ford, 1974) and since in stage 6 oocytes it was not possible to show poly(A)$^+$RNA of nuclear origin in the cytoplasm it would seem that this elevated hnRNA synthesis is not directed towards accumulation or translation of poly(A)$^+$RNA. The possibility that some of this hnRNA synthesis is directed towards the accumulation of repetitive sequence transcripts has been

proposed (Davidson et al., 1966; Crippa et al., 1967; Davidson, 1976) but it could also be that unstable mRNA molecules containing little or no poly(A) are produced from this hnRNA which are required for the synthesis of significant quantities of proteins to be stored during oogenesis. Certainly mRNA lacking poly(A) does appear to be unstable when injected into stage 6 oocytes (Marbaix et al., 1975) and a continuing synthesis might be required. In this connection, Anderson & Smith (1977) reported that in stage 6 Xenopus laevis oocytes about 15% of the stable RNA in the cytoplasm was heterogeneous RNA (4-40S) accumulating at a rate of about 100 pg/oocyte/hour (half-life > 90 hours) and was thought to be derived from the hnRNA. However, the method used to quantitate the data (Girard et al., 1965) is likely to suffer from both large and systematic errors depending on the accuracy of the base line and the predominance of rRNA over the heterogeneous RNA (Girard et al., 1965; Leoning et al., 1969). Further, since no deduction of mtRNA synthesis (up to 45 pg/oocyte/hour) and perhaps 4S and 5S RNA synthesis appears to have been made, both the rate of synthesis and the half-life of this putative cytoplasmic RNA are likely to be overestimates. However, it is still possible that some heterogeneous RNA does enter the cytoplasm and may be translated into protein, but if so this RNA would not appear to have sufficiently long 3' poly(A) sequences to allow isolation on oligo (dT)-cellulose (Chapter 4). Indeed, in stage 1 oocytes radioactive heterogeneous RNA was observed in the cytoplasm (Thomas, 1974), all of which cannot be accounted for by the poly(A)$^+$RNA known to be synthesized in these oocytes (Chapter 5).

It is also possible that the hnRNA synthesized during oogenesis is a precursor of stable poly(A)$^-$mRNA. In the case of histone mRNA it

has been shown that at all stages of oogenesis in Xenopus laevis histone mRNA is an abundant component (Ruderman & Pardue, 1977) about half of which is polyadenylated (Woodland, personal communication). Also estimates of the mRNA content of oocytes not based on properties of the poly(A) (Davidson & Hough, 1971; Hough & Davidson, 1972) give values which are higher than those based on techniques which involve poly(A) (Rosbash & Ford, 1974).

(g) Transcription rates

Estimates of the nucleotide "step time" or chain growth rate have been reported for RNA synthesis in Xenopus laevis oocytes and are between 15 and 30 nucleotides/second (Scheer, 1973; Davidson, 1976). It is instructive to generate values for this parameter using the rates of RNA synthesis obtained or estimated in this investigation and in Table 7-1 these are presented.

Knowing the number of copies of genes present in oocytes (Chapter 1) the rate of synthesis in nucleotides/second can be expressed as nucleotides/second/gene. If the RNA polymerase packing on the genes can be estimated then a value for the "step time" can be calculated. Approximately 100 transcripts are visible on each of the nucleolar genes of Xenopus laevis (Miller & Beatty, 1969b) and consequently "step times" of 2 nucleotides/second and 11 nucleotides/second are worked out using in vitro and in vivo rates respectively. If the same "step time" applies for the nucleolar RNA polymerase I in stage 1 oocytes then either 0.16% of the rRNA genes are fully active with about 100 polymerase molecules each, or if all the rRNA genes are available for transcription there is on average only one polymerase molecule for every 6 rRNA genes.

A different RNA polymerase, polymerase III, is thought to be responsible for transcription of 4S and 5S RNA generally (Lewin, 1974) and this is also true in Xenopus oocytes (Roeder, 1974). The small size of 4S and 5S genes would mean that at any one time only about 1 or 2 RNA polymerase molecules could be transcribing any particular gene. And thus "step times" of over 100 nucleotides/second per polymerase are calculated. Since in stage 6 oocytes both the nucleolar polymerase I and the low molecular weight polymerase III are probably transcribing at close to their maximum possible rates this difference in "step time" is suggestive of a difference in these two enzymes perhaps related to transcription control mechanisms.

For poly(A)$^+$RNA using 1.3×10^4 as the estimated number of genes (Davidson, 1976) and about 25 polymerase II molecules per gene region (i.e. similar packing to rRNA genes) a credible "step time" of about 1 nucleotide/second per polymerase is obtained for stage 1 oocytes but using similar parameters for stage 6 oocytes produces a "step time" of about 50 nucleotides/second per polymerase, which although possible is much higher than that for the nucleolar polymerase I. However, if less than 30% of the poly(A)$^+$RNA synthesized in stage 6 oocytes is nuclear in origin the corresponding "step time" would be <15 nucleotides/second which suggests that polymerase II resembles more closely the nucleolar polymerase I than it does polymerase III at least in terms of the maximum speed of transcription. A much lower "step time" would also be obtained if the mitochondrial gene number of 1.8×10^8 was used.

It was not possible to calculate "step times" for hnRNA synthesis since no reliable gene numbers were available.

(h) Lampbrush chromosomes

During oogenesis lampbrush chromosomes extend at about the time rRNA synthesis begins (Dumont, 1972; Davidson, 1976) and similar transcription units are visible in the electron microscope for both lampbrush chromosomal DNA and nucleolar DNA (Miller & Beatty, 1969a & b; Angelier & Lacroix, 1975). The mature oocyte contains vast quantities of all 3 RNA polymerase activities, equal to that of a 60,000 cell gastrula in Xenopus (Wasserman et al., 1972; Roeder, 1974), and Gurdon (1974) has proposed that the simplest model to account for the regulation of rRNA synthesis is one in which the rDNA is usually permanently available for transcription. In view of these observations and since in this investigation and elsewhere (Davidson, 1976) no positive function of the lampbrush chromosomes has been demonstrated it is tempting to speculate that lampbrush chromosomes are formed passively in response to changes in the oocyte cytoplasm. For example if the RNA polymerase concentration rises in the oocyte cytoplasm then in de-repressed genes initiation may take place more frequently and if as was suggested for the regulation of rRNA synthesis (Ford, 1972) a factor is required for the release of the transcript, it only requires that this factor becomes limiting (or transcript release itself) for there to be an increase in the packing ratio on the DNA. It is probably impossible for steric reasons for the DNA to remain condensed as the packing ratio increases. Thus the DNA extension seen in the ribosomal genes and in the lampbrush chromosomes may be due indirectly to the increases in the RNA polymerase concentrations occuring during oogenesis. To explain the collapse of the lampbrush chromosomes either much of the RNA polymerase is sequestered or prevented from initiating so rapidly. Thus depending on the relative rates of

initiation and transcript release after chromosome condensation, less RNA synthesis could occur as in Triturus or an equal amount as in Xenopus. This passive formation of lampbrush chromosomes would solve the problem outlined earlier in the text that lampbrush chromosomes are not necessary structures for the rapid synthesis of RNA and indeed the "step times" calculated in Table 7-1 suggest that for rRNA synthesis, despite a high packing ratio, the nucleolar RNA polymerase I is working slowly relative to the 4S and 5S RNA polymerase III. The "step time" for polymerase II also seems low relative to polymerase III and this may be due not just to differences between polymerases I and II and polymerase III but also to limiting transcript release from rRNA and hnRNA genes.

(i) Comparison of RNA synthesis in oocytes with that in somatic cells

Many of the features of RNA synthesis in oocytes so far discussed are similar to those occuring in somatic cells of animals, but there are a number of aspects which appear to be specific to oocytes and have no counterparts in somatic cells.

In a number of organisms (nematodes, midges) the existence of germ-line specific DNA or germ-line specific gene expression has been reported (Geyer-Duszynska, 1966; Bantock, 1970; Moritz & Roth, 1976) and in some of these cases, during embryogenesis somatic cells undergo chromosome diminution and it is therefore probable that in the germ cells RNA synthesis occurs on these chromosomes. In Xenopus it has been shown that although all the 5S RNA genes are present in all cells, the oocytes synthesize both the somatic and additional oocyte specific 5S RNA sequences (Ford & Southern, 1973).

The sequence organisation of the chromosomal DNA and of the hnRNA transcripts in both oocytes (Davidson et al., 1973; Sommerville & Malcolm, 1976) and a number of other animal cells (Holmes & Bonner, 1974; Molloy et al., 1974; Smith et al., 1974) shows an interspersed repetitive and non repetitive structure.

In mammalian cells the hnRNA transcripts become complexed with various proteins and the resulting hnRNP particles appear to be composed of sub-particles (Kinniburgh et al., 1976). It would appear that due to their length the RNA molecules must span a number of sub-particles. Sommerville (1973) has reported hnRNP particles with similar properties in the oocytes of Triturus. Base composition analysis of hnRNA from oocytes and mammalian cells have shown a DNA like base composition, a high uridylic acid content (Sommerville, 1973; Kinniburgh et al., 1976) and poly(A) tracts (Edmonds et al., 1976; Rogers & Browder, 1977b). Oligo (U) tracts are present in hnRNA from mammalian cells (Edmonds et al., 1976; Kinniburgh et al., 1976) and a fraction of both the nuclear poly(A) and oligo (U) is known to enter the cytoplasm in these cells (Edmonds et al., 1976). In other somatic cells some hnRNA molecules have been shown to have 5' "cap" structures the synthesis of which bears a precursor product relationship to cytoplasmic mRNA 5' "caps" which suggests that mRNA may be cleaved from the 5' end of some hnRNA molecules (Perry et al., 1976). While these particular nucleo-cytoplasmic relationships have not yet been demonstrated in oocytes, it does seem that hnRNA in both animal cells and oocytes contains sequences which are homologous to mRNA sequences (Lewin, 1975; Perry et al., 1976; Sommerville & Malcolm, 1976).

A very specific feature of oocytes are the lampbrush chromosomes on which active transcription is visible. These structures, which do

not appear to have a counterpart in somatic cells, have been discussed earlier. Also discussed earlier were the extremely rapid rates of RNA synthesis and high RNA polymerase concentrations observed in later oocyte stages and these three features which are probably related distinguish oocytes from other cell types where the rate of RNA synthesis may be more than 10^3 fold lower (Anderson & Smith, 1977). Indeed, Brandhorst & McConkey (1974) measured absolute rates of RNA synthesis in mouse L cells and obtained values of 3.2 pg/cell/hour for hnRNA synthesis and 0.072 pg/cell/hour for mRNA synthesis with average half-lives for these RNA species of about $\frac{1}{2}$ and 10 hours respectively. By comparison with the values in Table 7-1 for oocytes these rates are up to 500 times lower and this is thought to be due mainly to the higher RNA polymerase packing observed in oocytes (Anderson & Smith, 1977).

At least some of the cytoplasmic mRNA in both oocytes and somatic cells is present complexed with protein in the form of mRNP particles (Rosbash & Ford, 1974), but the proteins present and the protein:RNA ratios are different in these cell types (Darnbrough, personal communication). The distribution of cytoplasmic mRNA between mRNP particles and polysomes are also markedly different in these two cell types. In full grown Xenopus oocytes over 90% of the cytoplasmic poly(A)$^+$mRNA is in mRNP particles (Rosbash & Ford, 1974) whereas in typical somatic cells only about 10% of the cytoplasmic mRNA is in mRNP particles. The mRNA in mRNP particles in oocytes appears not to be active in protein synthesis until early embryogenesis, which suggests that mRNA can exist in a masked form in oocytes, (Humphreys, 1971; Gross et al., 1973; Gurdon, 1974) and masked mRNA may also be present in other animal cells (Spirin, 1966).

The oocyte is specialized for the accumulation of various cellular components and as a result its pattern of RNA synthesis differs from that in somatic cells. Firstly, the dramatic accumulation of mitochondria means that later in oogenesis mtRNA synthesis accounts for a measurable proportion of the total RNA synthesis (Webb et al., 1975). Secondly, the accumulation of rRNA throughout oogenesis means that significant differences occur in the relative proportions of the various RNA classes at different times during oogenesis. As shown in Table 7-1 the synthesis of stable RNA changes from being predominantly 4S and 5S RNA synthesis in stage 1 oocytes to being almost completely 18S and 28S RNA synthesis in stage 6 oocytes and the magnitude of this change is largely due to the amplification of the 18S and 28S RNA genes which does not occur in somatic cells. In somatic cells, only during mitosis are dramatic changes in the relative proportions of the various classes of RNA synthesis observed. 18S and 28S RNA synthesis appears to cease while tRNA and 5S RNA continue at a reduced rate (Zylber & Penman, 1971).

A third feature of the accumulation of cellular components during oogenesis is the exceedingly high stability of the RNA synthesized. As mentioned earlier, at various stages of oogenesis half-lives of 6 months to over 2 years have been reported for rRNA, 4S RNA, 5S RNA and poly(A)$^+$RNA (Anderson & Smith, 1977; Ford et al., 1977). This stability is likely to be primarily due to the oocyte cytoplasm (Ford et al., 1977) an environment in which mRNA, a classically unstable species of RNA, has a half-life greatly in excess of those recorded for typical somatic cells (Kafatos, 1972; Singer & Penman, 1973; Perry et al., 1976).

In various somatic cells 2 main classes of mRNA have been observed, one with a shorter half-life (< 5 hours) and the other with a longer half-life (about 20 hours) (Singer & Penman, 1973; Brandhorst & McConkey, 1974; Perry et al., 1976) but no specific functions have been assigned to these different classes. While it is difficult to explain, on the basis of present knowledge, how differences in mRNA stability arise, it is possible to speculate that the differences may be due to the position within the cytoplasm at which the mRNA is translated. Some regions of the cytoplasm may have lower concentrations of hydrolytic enzymes and mRNAs being translated or stored in this region may be more stable. Speculations of this type may require the cytoplasm to have a higher order of structure than previously considered and recent work suggests that this may be the case (Herman et al., 1976). To what extent the cytoplasmic architecture is responsible for the stability of oocyte RNA remains unknown.

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Differential Accumulation of Two Size Classes of Poly(A) Associated with Messenger RNA during Oogenesis in *Xenopus laevis*

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The RNA of full-grown oocytes of *Xenopus laevis* contains two distinct size classes of poly(A), designated poly(A)$_S$ and poly(A)$_L$, which contain 15–30 (mean = 20) and 40–80 (mean = 61) A residues, respectively. Both poly(A)$_L$ and poly(A)$_S$ are associated with RNA which is heterogeneous in size. The two classes of poly(A)$^+$ RNA can be separated by affinity chromatography: Only poly(A)$_L^+$ RNA binds to oligo(dT)-cellulose under appropriate conditions, but up to 50% of the poly(A)$_S^+$ RNA can be isolated from the void fraction by binding to poly(U)-Sepharose. Both classes of poly(A)$^+$ RNA are active as messenger RNA in an *in vitro* system and yield identical patterns of *in vitro* protein products. Previtellogenic oocytes contain almost exclusively poly(A)$_L$, which accumulates up to vitellogenesis but remains almost constant in amount (molecules/oocyte) during vitellogenesis and in the full-grown oocyte. Poly(A)$_S$ accumulates (molecules/oocyte) from early vitellogenesis up to the full-grown oocyte. The total number of poly(A)$^+$ RNA molecules per oocyte increases throughout oogenesis from 2×10^{10}/previtellogenic oocyte [80–90% poly(A)$_L$] to 20×10^{10}/full-grown oocyte (25–40% poly(A)$_L$). It is argued that poly(A)$_S$ is protected from degradation in the oocyte, thus stabilizing the "maternal" poly(A)$^+$ mRNA.

INTRODUCTION

The bulk of messenger RNA molecules in eukaryotes carry a 3′-terminal poly(A) sequence which shows a wide variation in length between cell types and according to the metabolic state of the cell. Newly synthesized mRNA in tissue culture cells carries a poly(A) chain of length $(A)_{150-200}$ (Kates, 1970; Nakazoto *et al.*, 1973), whereas the steady-state poly(A) population of many cell types shows a distribution of poly(A) chain lengths from $(A)_{40}$ to $(A)_{150}$ (Greenberg and Perry, 1972; Pemberton and Baglioni, 1972; Sheiness and Darnell, 1973; Gorski *et al.*, 1974; Greenberg, 1975). Certain mRNA populations carry shorter poly(A) chains in the range $(A)_{20}$–$(A)_{40}$; examples are yeast mitochondrial mRNA (Hendler *et al.*, 1975), insect chorion mRNA (Vournakis *et al.*, 1974), *Dictyostelium* mRNA (Jacobson *et al.*, 1974), and a proportion of bacterial mRNAs (Nakazoto *et al.*, 1975; Otha *et al.*, 1975). In certain cases, the decrease in size of the poly(A) chain can be related to aging in the messenger RNA (Sheiness and Darnell, 1973; Gorski *et al.*, 1974).

Xenopus laevis oocytes contain a store of poly(A)$^+$ RNA which, in the full-grown oocyte, is largely associated with ribonucleoprotein particles (Rosbash and Ford, 1974), but which is nevertheless active as messenger RNA in an *in vitro* system (Darnbrough and Ford, 1976). Rosbash and Ford (1974) determined the steady-state size of the poly(A) sequences in the full-grown oocyte and found a mean size of approximately $(A)_{100}$. Determinations of the amount of poly(A) per oocyte at different stages of oogenesis led to the conclusion that poly(A)$^+$ RNA accumulates up to

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early vitellogenesis and, thereafter, remains constant in amount until fertilization. Since steady-state levels of poly(A) were measured, no information was gained about the rates of synthesis and turnover of poly(A)$^+$ RNA.

We now report the identification of two classes of poly(A)$^+$ RNA which differ in the length of the poly(A) sequence, but which have comparable activities in the stimulation of protein synthesis in an *in vitro* system and yield apparently identical patterns of protein products. We demonstrate that the two classes of poly(A)$^+$ RNA accumulate during different stages of oogenesis, and that accumulation of poly(A)$^+$ RNA (mRNA) continues throughout oogenesis. The results are discussed in terms of the stability of mRNA during the prolonged later stages of oogenesis.

MATERIALS AND METHODS

Materials. Xenopus laevis toads were obtained from the South African Snake Farm, Fish Hoek, South Africa. Radiochemicals were from the Radiochemical Centre, Amersham. All common reagents were Analar grade wherever possible. Inorganic buffers were sterilized with diethylpyrocarbonate.

Preparation and fractionation of isolated oocytes. Oocytes were released from the ovary by treatment of ovary fragments with 10 mg/ml of collagenase in modified Barth X + 20 m*M* EDTA, pH 7.5. After 20–30 min of gentle agitation, the dissociated oocytes were washed several times in fresh Barth X (Barth and Barth, 1959) and were sorted manually into the stages described by Rosbash and Ford (1974): (I) clear, previtellogenic; (II) white, early vitellogenic; (III) brown, small pigmented; (IV) half-size; (VI) full-grown. All stages of oocytes were prepared from mature ovaries, but clear oocytes were sometimes prepared in the same way from small immature ovaries. Occasionally, full-grown oocytes were prepared simply by plucking them with forceps from the untreated ovary; this method does not remove the follicle cells.

Extraction of RNA. RNA was prepared from oocytes by homogenizing the oocytes in modified Kirby buffer (Kirby, 1965) at pH 9.0 and extracting with an equal volume of phenol/cresol (6:1) or phenol/chloroform (1:1) as described by Darnbrough and Ford (1976), followed by ethanol precipitation.

Affinity chromatography using oligo-(dT)-cellulose and poly(U)-Sepharose. Columns of oligo (dT)-cellulose (Collaborative Research Inc., grade T-3) of 1-ml bed volume in 2-ml plastic syringes were run at a flow rate of 0.5–1.0 ml/min. Between samples, columns were regenerated with and stored in 0.1 *M* NaOH. The RNA sample dissolved in binding buffer (0.4 *M* NaCl, 1 m*M* EDTA, 0.1% SDS, 10 m*M* Tris–HCl, pH 7.5) was passed twice through the pre-equilibrated column, followed by 2 × 2 ml of binding buffer, and the void fraction (V) was collected. Bound RNA was eluted with 3 × 1 ml of elution buffer (1 m*M* EDTA, 0.1% SDS, 10 m*M* Tris–HCl, pH 7.5). The bound fraction was made up to 0.4 *M* NaCl and was passed through the column once more, followed by 2 ml of buffer containing 0.15 *M* NaCl, and was eluted with elution buffer to give a bound-bound fraction (BB).

Poly(U)-Sepharose was prepared by linking poly(U) (Sigma, Type II) to cyanogen bromide-activated Sepharose 4B as described by Wagner *et al.* (1971). The product contained 1–2 mg of poly(U)/1-ml bed volume of Sepharose and was stored under elution buffer (see ahead) at −20°C. Columns of 0.5-ml bed volume were formed in 2-ml plastic syringes and were washed with 10 column vol of elution buffer (50% deionized formamide in 1 m*M* EDTA, 1 m*M* Tris–HCl, pH 7.0, 0.05% SDS) and 10 col volumes of binding buffer (as for oligo(dT)-cellulose) before use. The sample (usually the void from oligo(dT)-cellulose) dissolved in binding buffer was passed through the column twice at room temperature, followed by 5 ml of binding buffer,

to give the void fraction. Bound RNA was eluted with 5 × 1 ml of elution buffer at room temperature (22–25°C). We consistently found that a small amount of free poly(U) was washed off the column into all buffers, amounting to 0.2–0.5 μg of poly(U)/5 ml of buffer.

Yields of poly(A) were estimated after ethanol precipitation by [^{3}H]poly(U) binding assays (see ahead). For poly(U)-Sepharose fractions, a dilution assay was used because the free poly(U) competes with the [^{3}H]poly(U) in the assay.

[^{3}H]Poly(U) binding assay. Poly(A) was estimated by measuring the amount of [^{3}H]poly(U) rendered ribonuclease-resistant by annealing to the RNA (Bishop *et al.*, 1974), as described in Darnbrough and Ford (1976). The poly(U) had a specific activity of 600,000 or 700,000 cpm/μg of poly(A). The values quoted in figures have been corrected for (minus sample) controls and specific activity.

Wheat germ S-30 cell-free system. The preparation of the wheat germ extract and the conditions for protein synthesis were exactly as described in Darnbrough and Ford (1976). Incubations were carried out at 25°C. The products of mRNA-directed protein synthesis were analyzed by polyacrylamide gel electrophoresis, using the buffer system of Laemmli (1970) on slab gels as described by Darnbrough and Ford (1976). The [^{35}S]methionine-labeled products were detected by autoradiography as described.

Ribonuclease T_1 digestion of RNA. Poly(A) was prepared from RNA samples by digestion with DNase and RNase T_1. An amount of RNA (1–40 μg) containing approximately 10,000 cpm of [^{3}H]poly(U) binding was dissolved in 0.2–1 ml of a buffer containing 0.1 *M* NaCl, 1 m*M* $MgCl_2$, 10 m*M* Tris–HCl, pH 7.0, and was treated with 40 μg/ml of DNase I (Sigma, electrophoretically pure) for 30 min at 37°C. EDTA was added to 10 m*M*, followed by RNase T_1 (Sigma) to give a RNA:RNase ratio of 20:1 (w/w). After a further 30 min at 37°C, the mixture was chilled on ice, and RNA was precipitated by adding 2.5 vol of ethanol and 10–50 μg of *E. coli* RNA as carrier. This carrier contained 8 cpm/μg of [^{3}H]poly(U) binding which was corrected for when examining the size distribution of the poly(A) on gels (see ahead).

Polyacrylamide gel electrophoresis of poly(A). Cylindrical gels, 9 cm long, contained 10/0.25% acrylamide/bisacrylamide in 36 m*M* Tris, 30 m*M* NaH_2PO_4, 2 m*M* EDTA, pH 7.8, and were prerun for 30 min at 7 mA/gel. The sample, prepared by RNase T_1 digestion as described and dissolved in gel buffer plus 0.2% SDS, 10% glycerol, and bromophenol blue, was applied to the gel, and electrophoresis was continued at 7 mA/gel until the bromophenol blue was approximately half-way down the gel. Marker gels, with 4S and 5S RNAs, were run in parallel and were scanned using a Gilson spectrophotometer with a gel scanning attachment. The sample gels were sliced with a Mickle gel slicer, and fractions of two adjacent 1-mm slices were homogenized in 1 ml of 2× SSC and were incubated for 30 min at 37°C to elute the poly(A) before spinning out the acrylamide. The pellet was washed with a further 0.5 ml of 2× SSC, and the poly(A) was thus recovered in a volume of 1.2 ml/fraction. An excess of [^{3}H]poly(U) was added to each fraction and the [^{3}H]poly(U) binding was determined as described before. The total yield of poly(U) binding was checked for each gel and was normally within the range 85–110% of input. Correction was made for the poly(A) added in the *E. coli* RNA carrier, which migrated with a size of $(A)_{20-30}$. In general, this correction was insignificant.

RESULTS

Calibration of SDS–Polyacrylamide Gels

For quantitative and technical reasons it has not been possible to prepare oocyte [^{3}H]adenosine-labeled poly(A) in the amounts required to measure oocyte poly(A) sizes directly by determination of

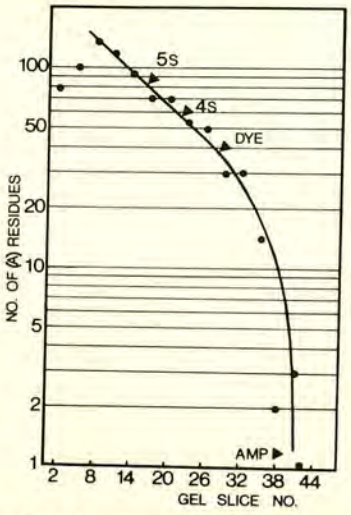

FIG. 1. Poly(A) size calibration of polyacrylamide gels by estimation of [^{3}H]AMP:adenosine ratio. HeLa cells in spinner culture were labeled for 48 hr with 50 μCi/ml of [^{3}H]adenosine, and total RNA was obtained by phenol extraction at pH 9. The RNA was digested with DNase and RNase T_1 as described in Materials and Methods, and poly(A) was isolated by oligo(dT)-cellulose chromatography. The ^{3}H-labeled poly(A) was electrophoresed on 10% polyacrylamide gels (see Materials and Methods), with 4S and 5S RNAs and bromophenol blue on the same gel, for 4 hr at 7 mA/gel until the dye had migrated about halfway through the gel. Gels were scanned at 265 nm to localize the markers and were cut into 2-mm slices: poly(A) was eluted from each slice, and aliquots were determined by scintillation counting. These fractions were precipitated with 100 μg of carrier RNA and 2.5 vol of ethanol at −20°C overnight, and the RNA pellets were dissolved in 0.2 *M* NaOH and were incubated at 37°C overnight. Samples were spotted onto Whatman No. 52 paper and were electrophoresed in 7% acetic acid/pyridine, pH 3.5, at 3 kV for 45 min. AMP and adenosine (as uv markers) and [^{3}H]adenosine (for a recovery assay) were run in parallel. The sample tracks were cut out and 1-cm slices were determined by scintillation counting on the paper in toluene/butyl-PBD, covering the region from the adenosine marker to UMP. Under these conditions, adenosine runs to the anode; CMP, AMP, GMP, and UMP run to the cathode in order of increasing mobility. Paper slices containing radioactivity were eluted extensively with distilled water and dried down under vacuum, and ^{3}H radioactivity was estimated by scintillation counting in toluene/butyl-PBD. No radioactivity was associated with CMP, GMP, or UMP, but 2–5% of the total radioactivity remained at the origin. The length of the poly(A) on a given track or gel fraction, the AMP:A_{OH} ratio. Therefore, poly(A)$^+$ RNA from HeLa cells labeled with [^{3}H]adenosine for 48 hr was digested with T_1 ribonuclease and was analyzed on a 10% polyacrylamide gel (see Materials and Methods). The gel was sliced, poly(A) was eluted from groups of slices, and its size was determined by hydrolysis and estimation of the [^{3}H]AMP:[^{3}H]adenosine ratio by paper electrophoresis at pH 3.5. From the calibration curve shown in Fig. 1, we assigned values for poly(A) size to the positions of our markers, 5S RNA, 4S RNA, and bromophenol blue, of $(A)_{82}$, $(A)_{58}$, and $(A)_{38}$, respectively. These values were used to estimate poly(A) sizes in this and subsequent experiments using the identical gel system.

Poly(A) and Poly(A)$^+$ RNA in Full-Grown Oocytes

The size distribution of poly(A) prepared from full-grown oocyte RNA by digestion with DNase and T_1 RNase is shown in Fig. 2d. Two distinct size classes were present, which we have designated $poly(A)_L$ and $poly(A)_S$ and which were estimated to contain, respectively, $(A)_{40}$–$(A)_{80}$ [mean$(A)_{60}$] and $(A)_{15}$–$(A)_{30}$ [mean$(A)_{20}$] (means of 30 experiments). $Poly(A)_L$ is equivalent to the oocyte poly(A) described by Rosbash and Ford (1974), who failed to observe the smaller poly(A) species probably because it migrates faster than the bromophenol blue in our gel system. The size of $poly(A)_S$ cannot be regarded as accurate since the slope of the calibration curve is very steep in this region. However, the two classes of poly(A) are not artifactual since they can be separated using oligo(dT)-cellulose (see ahead) and since their relative amounts vary in a consistent way during oogenesis (see later). The poly(A) size profile was not altered when the RNA was digested with

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$poly(A)_n$, was calculated from the formula:

$$n = (\text{cpm in AMP} - \text{background}/\text{cpm in adenosine} - \text{background}) + 1.$$

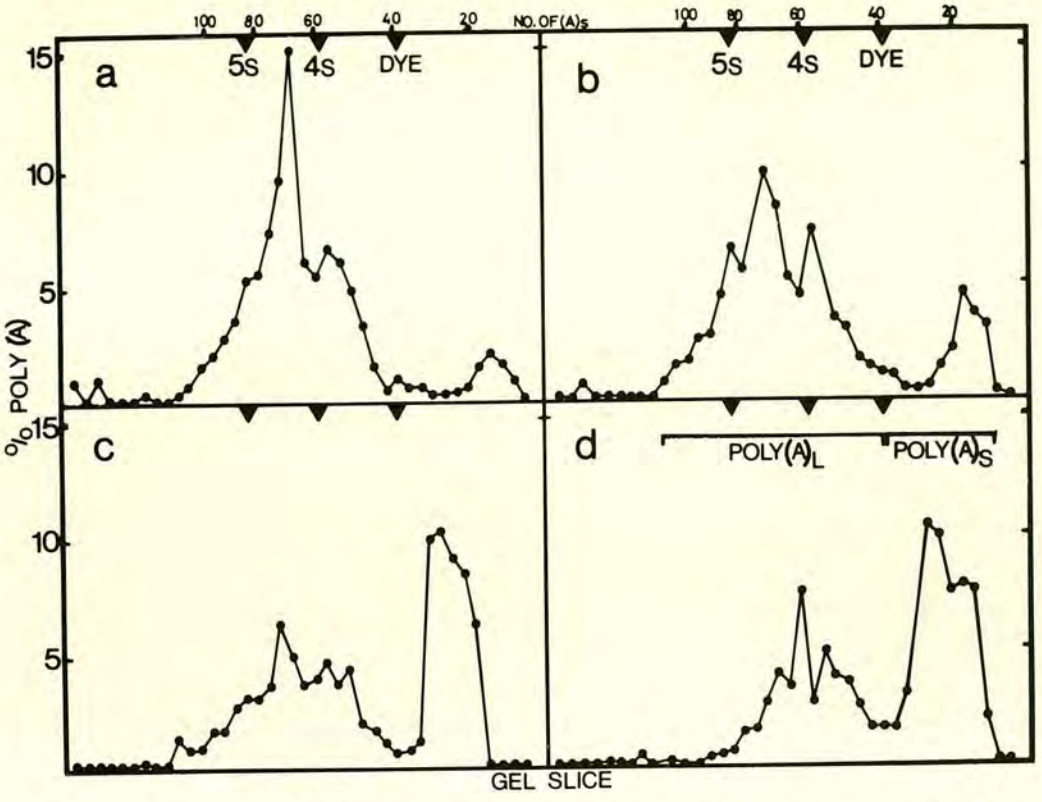

FIG. 2. Size distributions of poly(A) from isolated oocytes. Follicle-free oocytes were prepared from mature ovaries and were separated into stages, and RNA was extracted as described in Materials and Methods. Amounts of RNA containing approximately 10,000 cpm of [^{3}H]poly(U) binding were digested with DNase and RNase T_1, and the poly(A) was analyzed by polyacrylamide gel electrophoresis, followed by [^{3}H]poly(U) binding assays on the sliced gels. Recovery of [^{3}H]poly(U) binding from the gels was 90–105% of the total counts per minute before digestion, after correction for 400 cpm of [^{3}H]poly(U) binding from the *E. coli* RNA carrier. 4S and 5S RNA markers were run on parallel gels; from the positions of the markers, Fig. 1 was used to estimate the poly(A) sizes shown at the top of the figure. The figure shows the percentage per fraction of recovered [^{3}H]poly(U) binding. (a) Stage I (previtellogenic) oocytes; (b) stage II (early vitellogenic) oocytes; (c) stage IV (half-size) oocytes; (d) stage VI (full-grown) oocytes.

both pancreatic ribonuclease and T_1 ribonuclease.

Separation of Poly(A)$_L^+$ RNA and Poly(A)$_S^+$ RNA by Affinity Chromatography

The two classes of poly(A)$^+$ RNA can be resolved almost completely using oligo(dT)-cellulose. As shown in Fig. 3b, the RNA bound to oligo(dT)-cellulose contained essentially only poly(A)$_L$ with a mean size of $(A)_{68\pm5}$, whereas the unbound RNA (Fig. 3a) contained only poly(A)$_S$. This separation was reproducible; under these conditions, each class of poly(A)$^+$ RNA was contaminated with 10–15% of the other. After passing the unbound RNA through poly(U)-Sepharose, elution with 50% formamide yielded 30–50% of the remaining poly(A), which was greatly enriched for poly(A)$_S$ with a mean size of $(A)_{38\pm4}$ (Fig. 3c). The details of this fractionation procedure are shown in Table 1.

Sucrose gradient centrifugation of oligo(dT)-cellulose and poly(U)-Sepharose bound fractions (Fig. 4a,b) showed that both poly(A)$_L$ and poly(A)$_S$ are associated with RNA which is heterogeneous in size,

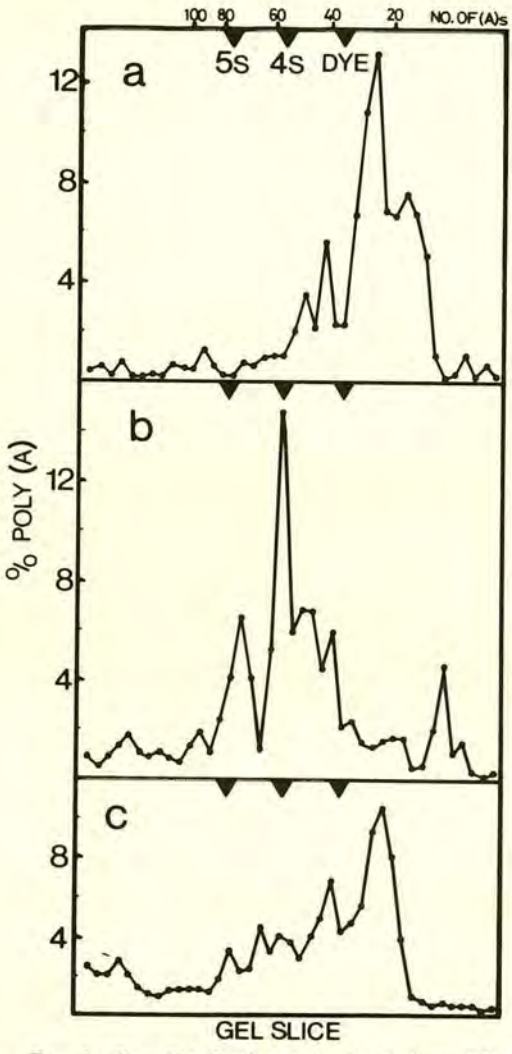

FIG. 3. Size distributions of poly(A) from RNA from full-grown oocytes after fractionation on oligo(dT)-cellulose and poly(U)-Sepharose columns. Total RNA from full-grown oocytes was fractionated on oligo(dT)-cellulose and poly(U)-Sepharose as detailed in Table 1. The RNA fractions were digested with DNase and RNase T_1 and were analyzed on polyacrylamide gels, followed by [^{3}H]poly(U) binding assays. Recovery of [^{3}H]poly(U) binding from the gels was 90–100% of the counts per minute before digestion, after correction for 100 cpm from *E. coli* carrier RNA. Poly(A) sizes were estimated using Fig. 1, from the positions of 4S and 5S RNA markers run on parallel gels. The figure shows the percentage per fraction of recovered [^{3}H]poly(U) binding; the actual counts recovered are shown in parentheses in the following: (a) oligo(dT)-cellulose void (27,000 cpm); (b) oligo(dT)-cellulose bound (19,900 cpm); (c) poly(U)-Sepharose bound (5600 cpm).

having mean sedimentation coefficients of 20S and 18S (approximately), respectively. The poly(U)-Sepharose unbound poly(A)$^+$ RNA (Fig. 4c) was also heterogeneous in size distribution. These results indicate that poly(A)$_S$ is not a component of 18S or 28S ribosomal RNA, but, like poly(A)$_L$, is associated with messenger-like RNA. Note also that the poly(U)-Sepharose bound RNA was contaminated with 18S and 28S RNAs, whereas none was detectable in the oligo(dT)-cellulose bound.

Translation of Poly(A)$^+$ RNA from Full-Grown Oocytes

Figure 5 shows the stimulation of protein synthesis obtained when oligo(dT)-cellulose bound and poly(U)-Sepharose bound RNAs were translated in the wheat germ cell-free system at a range of RNA concentrations. The RNA samples were prepared and characterized as detailed in Table 1 and Figs. 3 and 4. In order to correct for any possible effect on translation of the small amount of free poly(U) present in the poly(U)-Sepharose fractions, equivalent amounts of poly(U) were added to incubations containing oligo(dT)-bound RNA; the poly(U) was found to have no effect on the rate of protein synthesis.

Over the range of RNA concentrations used in the translation assay, the ratio of the template activities of poly(U)-Sepharose bound : oligo(dT)-cellulose bound RNAs was 0.40–0.45 for any given RNA concentration (from Fig. 5), resulting from greater contamination of the poly(U)-Sepharose bound fraction with rRNA (see Fig. 4). This compares with a ratio of 0.42 ± 0.08 for the relative molar concentrations of poly(A)$^+$ RNA in the two samples, estimated from the known values for poly(A) content (Table 1) and poly(A) size (Fig. 3). Thus, the template activities show that poly(A)$_L^+$ RNA and poly(A)$_S^+$ RNA are equally effective templates for *in vitro* protein synthesis.

The *in vitro* translation products were analyzed by polyacrylamide gel electro-

TABLE 1

FRACTIONATION OF FULL-GROWN OOCYTE RNA ON OLIGO(dT)-CELLULOSE AND POLY(U)-SEPHAROSE COLUMNS[a]

Fraction	RNA (μg)	Poly(A) (μg)	Recovery of poly(A) (%)	Poly(A) content (%)
Oligo(dT)-cellulose				
Load	3400	1.2	(100)	0.035
Void	3060	0.42	35	0.014
Bound-void	54	0.07	5.8	0.13
Bound-bound	27	0.68	57	2.5
Poly(U)-Sepharose				
Load [void from oligo(dT)]	3000	0.40	(100)	0.014
Void	2950	0.15	38	0.005
Bound	24	0.14	35	0.58

[a] Total RNA from full-grown oocytes was fractionated on oligo(dT)-cellulose and poly(U)-Sepharose as described in Materials and Methods. The void fraction from oligo(dT)-cellulose was loaded directly on to poly(U)-Sepharose. RNA was precipitated from each fraction by adding 0.1 vols of 2 *M* NaCl where necessary and 2.5 vol of ethanol at −20°C overnight. The amount of RNA was determined from the absorbance at 260 and 280 nm, and poly(A) was estimated by hybridization to [³H]poly(U) at a specific activity of 6×10^5 cpm/μg of poly(A), as described in Materials and Methods. The poly(U)-Sepharose fractions contained small amounts (0.5–1 μg) of free poly(U) from the column, and [³H]poly(U) binding was determined by a dilution method.

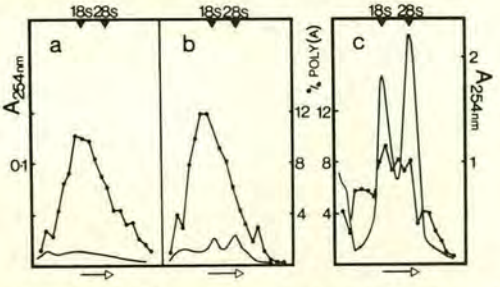

FIG. 4. SDS–Sucrose gradient centrifugation of poly(A)$_L$⁺ RNA and poly(A)$_S$⁺ RNA from full-grown oocytes. Full-grown oocyte RNA was fractionated on oligo(dT)-cellulose and poly(U)-Sepharose as detailed in Table 1, and aliquots of the fractions were analyzed by centrifugation on gradients of 7–30% sucrose in NETS buffer, spun for 4.5 hr at 40,000 rpm and 23°C in the MSE 6 × 14 ml Ti rotor. The gradients were pumped out through an ISCO uv analyzer and fractionated, and [³H]poly(U) binding was determined on aliquots of each fraction. Recovery of [³H]poly(U) binding was 80–90%. The figure shows the percentage of recovered [³H]poly(U) binding per fraction, overall recoveries in counts per minute per gradient are shown in the following. Centrifugation was from left to right. (a) Oligo(dT)-cellulose-bound RNA (60,000 cpm); (b) poly(U)-Sepharose-bound RNA (8500 cpm); (c) poly(U)-Sepharose-void RNA (4900 cpm). (—) A_{260}; (●—●) percentage of [³H]poly(U) binding recovered from gradient.

phoresis (Fig. 6). In a single dimension, no major differences were apparent between the products of poly(A)$_L$⁺ RNA and poly(A)$_S$⁺ RNA. Since we have shown previously that translation in the wheat germ system does reflect major differences between different mRNA populations (Darnbrough and Ford, 1976), we conclude that the two classes of poly(A)⁺ RNA are similar, at least in the high-abundance low-complexity class of mRNA readily detected by translation in the wheat germ system.

Accumulation of Poly(A)⁺ RNA during Oogenesis

We obtained values for total RNA and poly(A) content during oogenesis (Table 2) in agreement with those of Rosbash and Ford (1974). Total poly(A), measured by [³H]poly(U) binding, increased up to early vitellogenesis and thereafter remained almost constant.

The distribution of poly(A) between the two size classes just described was examined for five stages of oogenesis. Pre- and early vitellogenic oocytes contained

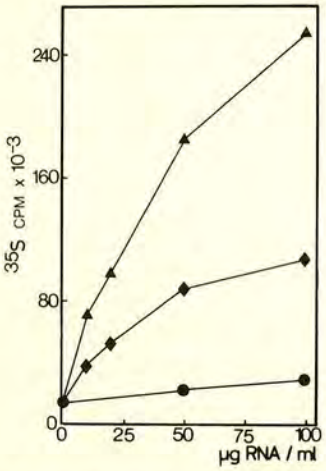

FIG. 5. *In vitro* translation of poly(A)$_L^+$ RNA and poly(A)$_S^+$ RNA. Full-grown oocyte RNA was fractionated on oligo(dT)-cellulose and poly(U)-Sepharose columns as detailed in Table 1, giving the bound poly(A)$^+$ RNA samples characterized in Figs. 3 and 4. The poly(A)$^+$ RNAs were added at various concentrations to wheat germ incubations containing 200 μCi/ml of [^{35}S]methionine and were incubated at 25°C. At intervals, 2-μl samples were withdrawn and assayed for incorporation of radioactivity into alkali-stable TCA-precipitable material. The rate of protein synthesis was linear for at least 60 min. The figure shows the counts per minute of [^{35}S]methionine incorporated into protein after 60 min, plotted as a function of RNA concentration. (▲—▲) Poly(A)$_L^+$ RNA [oligo(dT)-cellulose bound, 2.5% poly(A), mean size $A_{68\pm5}$]; (◆—◆) poly(A)$_S^+$ RNA [poly(U)-Sepharose bound, 0.58% poly(A), mean size $A_{38\pm4}$]; (●—●) poly(U)-Sepharose-void RNA (0.005% poly(A)].

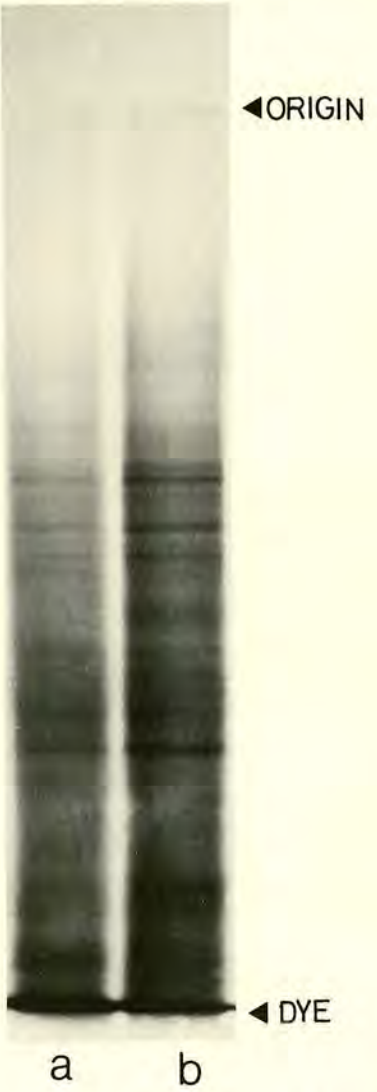

FIG. 6. SDS–Polyacrylamide gel electrophoresis of *in vitro* translation products of poly(A)$_L^+$ RNA and poly(A)$_S^+$ RNA. The wheat germ incubations in Fig. 5 were analyzed by electrophoresis on a 14% SDS–polyacrylamide gel (Laemmli, 1970). The gel was fixed in 30% MeOH/10% AcOH, dried down, and autoradiographed for 3 days. No bands were seen in the incubation without added RNA. Both RNA samples were added at a concentration of 100 μg/ml, and incubation was for 90 min at 25°C. (a) Poly(A)$_S^+$ RNA; (b) poly(A)$_L^+$ RNA.

largely poly(A)$_L$ (Fig. 2a,b), but vitellogenic stages contained an increasing proportion of poly(A)$_S$ (Fig. 2c,d). This suggests that, although the total amount of poly(A) per oocyte (Table 2) increased only slightly or not at all after early vitellogenesis, the total number of poly(A)$^+$ RNA molecules was increasing throughout oogenesis. Since both the molecular weight and the amount of poly(A) in each slice of the gels are known, the data can be expressed in terms of the number of poly(A) molecules per oocyte for each poly(A) size. This is shown in Fig. 7 for stages I (previtellogenic), II (early vitellogenic), III (early pigmented), and VI (full-grown) oocytes. The size distribution within each of the two poly(A) classes did not alter significantly through oogenesis, but, during the vitellogenic stages, the number of poly(A)$_S$

molecules per oocyte increased dramatically. The total numbers of molecules of each poly(A) class are plotted in Fig. 8 as a function of the amount of RNA per oocyte, which is probably a criterion better related to the timing of development than the cytological stages, shown by bars at the top of Fig. 8. The data from two animals are presented.

Note that the numerical values presented in Fig. 8 may be regarded as only approximate, since the processing of the data involves the following questionable assumptions: (1) that the poly(A) size determinations are accurate, (2) that ^3H poly(U) interacts with identical stoichiometry under all assay conditions with poly(A) molecules of all sizes, and (3) that

TABLE 2

RNA AND POLY(A) CONTENT OF OOCYTES DURING OOGENESIS[a]

Stage of oogenesis	Animal 1			Animal 2		
	(μg of RNA)	(ng of poly(A))		(μg of RNA)	(ng of poly(A))	
	1	2	3	1	2	3
I. Clear	0.2	1.4	0.7	0.1	2.3	0.8
II. White	1.3	5.6	2.1	0.7	6.1	2.4
III. Brown	2.6	4.3	2.3	2.3	5.9	2.4
IV. Half-size	4.2	4.8	2.5	3.2	6.7	2.5
VI. Full-grown	6.0	2.7	3.8	4.3	6.7	2.6

[a] Oocytes were isolated from the ovaries of two different animals and were sorted manually into the stages described by Rosbash and Ford (1974). RNA was extracted from known numbers of oocytes and RNA and poly(A) were estimated directly as described in Materials and Methods. These values are shown in columns 1 (μg of RNA/oocyte) and 2 (ng of poly(A)/oocyte). In addition, column 3 shows total recovery of poly(A)/oocyte after RNase digestion, polyacrylamide gel electrophoresis, elution from gel slices, and determination of $poly(A)_L$ and $poly(A)_S$ using the values shown in Fig. 8.

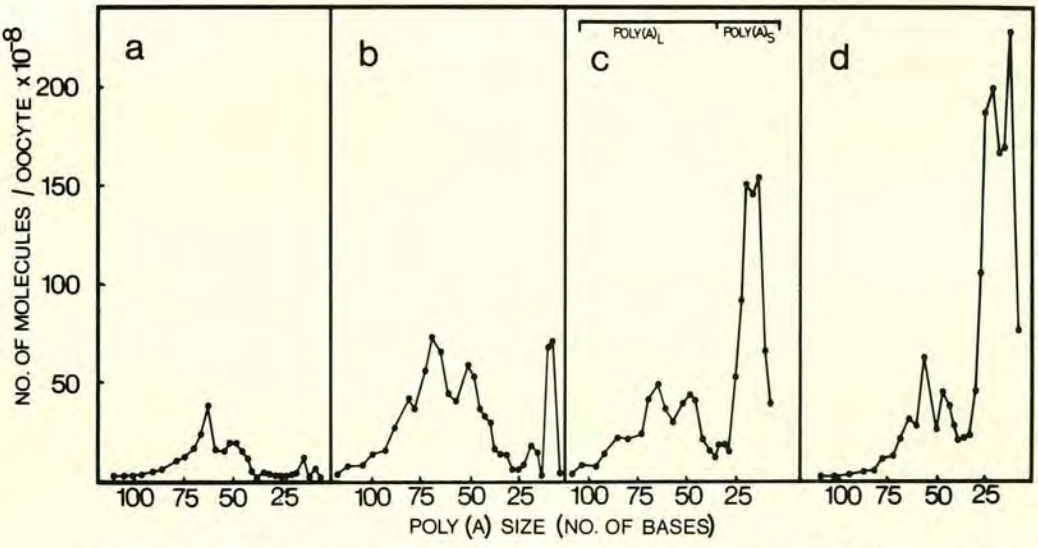

FIG. 7. Distributions of poly(A) sizes in terms of the number of molecules of poly(A) per oocyte during oogenesis. Poly(A) from counted numbers of oocytes of various stages was analyzed as described in Fig. 2. For each gel fraction, the poly(A) size was estimated using Fig. 1, and this value was used to convert the counts per minute of [^3H]poly(U) binding into the number of molecules of poly(A) per oocyte. The number of molecules of poly(A) are plotted here against a linear scale of the number of A residues per poly(A) molecule. (a) Stage I oocyte; (b) stage II; (c) stage III; (d) stage VI.

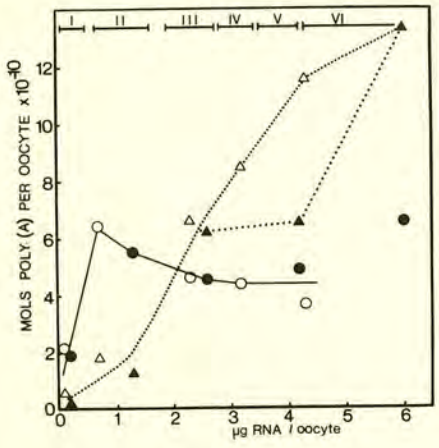

FIG. 8. Accumulation of poly(A)$_L$ and poly(A)$_S$ during oogenesis. Oocytes were isolated from the ovaries of two different animals: RNA was extracted and the amount of RNA was estimated as in Table 2. Poly(A) size distributions were determined as in Fig. 7. From these patterns the total numbers of molecules per oocyte of poly(A)$_L$ and poly(A)$_S$ were estimated and are plotted here as a function of the total RNA per oocyte. The approximate cytological stages are shown above the figure. Molecules of poly(A)$_L$ per oocyte $\times 10^{-10}$ (circles on solid line); molecules of poly(A)$_S$ per oocyte $\times 10^{-10}$ (triangles on dotted line); blank and darkened symbols represent different animals.

100% of the poly(A) is eluted from the gel slices. With the exception of the final value for animal 1, the values for total poly(A) weight per oocyte calculated from Fig. 8 are consistently 2–2.5 times smaller than the same values determined directly (Table 2, compare columns 2 and 3). This systematic error arises from the assumptions noted above and from systematic losses in the steps leading to Fig. 8. Nevertheless, within the likely limits of experimental error, both sets of data in Table 2 indicate that the total poly(A) weight per oocyte does not increase significantly during vitellogenesis.

We conclude that poly(A)$^+$ RNA accumulates throughout oogenesis, and that there is a well-defined change in the size of the poly(A) associated with messenger RNA which accumulates before and after the beginning of vitellogenesis.

DISCUSSION

In this report, we have demonstrated the existence of two distinct classes of poly(A)$^+$ RNA in *Xenopus* oocytes (Figs. 2, 3, and 8) which are similar in RNA size distribution and coding properties (Figs. 4–6). Since these classes accumulate during different periods of oogenesis they could represent mRNA populations which differ specifically in synthesis, stability, or function.

Structure of Poly(A)$^+$ RNA

Although we have not shown whether the smaller poly(A)$_S$ sequence is in the 3′-terminal position of the RNA molecule, we believe it to be associated with mRNA molecules having the same properties as those with poly(A)$_L$. Since the two classes of poly(A)$^+$ RNA can be separated using oligo(dT)-cellulose, they are distinct molecules, and, therefore, we can rule out the possibility of a poly(A)$_S$ sequence occurring within an mRNA molecule which also carries 3′-terminal poly(A)$_L$, such as is found in the mRNA of *Dictyostelium*, where the internal $(A)_{25}$ sequence is transcribed from the genome (Jacobson *et al.*, 1974). It is also likely that each poly(A)$_S^+$ molecule carries only one poly(A)$_S$ sequence since RNA molecules carrying $(A)_{60}$ and $(A)_{30}$ are completely separable on oligo(dT)-cellulose using our conditions and, therefore, must differ markedly in their affinity for the column. We explain this difference in affinity by considering the maximum chain length of oligo(dT), $(dT)_{20}$, and assuming that $(A)_{60}$ hybridizes to more than one oligo(dT) chain, whereas $(A)_{30}$ binds to only a single oligo(dT). We also assume that two or more $(A)_{30}$ chains within an RNA molecule should confer on it affinity for oligo(dT)-cellulose not less than that of a molecule carrying a single chain of $(A)_{60}$. Thus, we have no evidence to suggest that the two classes of poly(A)$^+$ RNA differ structurally in any respect other than that of poly(A) size.

Synthesis and Stability of Poly(A)⁺ RNA

In contrast to the earlier conclusion of Rosbash and Ford (1974), we have shown that poly(A)⁺ RNA accumulates through the vitellogenic stages of oogenesis at a rate which is proportional to the rate of ribosomal RNA accumulation. Since the onset of accumulation of poly(A)$_S$ coincides with the completion of ribosomal DNA amplification and the onset of ribosomal RNA synthesis, this abrupt change in poly(A) size might reflect a change in the length of poly(A) chain added post-transcriptionally. Experiments are in progress to determine the size of newly synthesized cytoplasmic poly(A); preliminary results indicate that both stage I and VI germinal vesicles contain poly(A) in the range (A)$_{100-150}$ (Darnbrough and Turner, unpublished), and it is probable that newly synthesized poly(A) is larger than poly(A)$_L$ or poly(A)$_S$ at all stages of oogenesis.

The observed accumulation of poly(A) sizes may, instead, result from a change in the cytoplasmic processing of poly(A). In other systems, poly(A) size has been correlated with the age of the mRNA (Sheiness and Darnell, 1973; Gorski *et al.*, 1974). The time scale of oogenesis is prolonged, the entire process requiring a minimum of about 3 months (Dumont, 1972), and the results of *in vivo* "pulse-chase" labeling experiments have indicated that previtellogenic poly(A)⁺ RNA [oligo(dT)cellulose bound] has a half-life in excess of 2 years (Ford *et al.*, 1977). As a result of these findings we suggest that poly(A)$_S$ and, perhaps, poly(A)$_L$ also are limit digests of poly(A) attached to stable RNA molecules, and that the accumulation of two relatively homogeneous poly(A) sizes results from the relative rates of mRNA production and poly(A) processing during oogenesis.

Allende *et al.* (1974) observed that 30–40% of the poly(A)⁺ RNA injected into oocytes was stable for at least 22 hr, but that poly(A)⁻ RNA was degraded rapidly. Other recent reports have shown that full translation stability of the globin mRNA injected into stage VI oocytes requires a poly(A) chain not shorter than (A)$_{12-32}$, globin mRNA with shorter poly(A) being translationally unstable (Huez *et al.*, 1975; Nudel *et al.*, 1976) and poly(A)⁻ globin mRNA being completely degraded (Marbaix *et al.*, 1975). Hieter *et al.* (1976) observed that 3′ poly(A) inhibits the degradation of RNA by RNase *in vitro*. In the light of these reports, we suggest that, in the oocyte the 3′ poly(A) of at least a proportion of the mRNA molecules is degraded only to a limiting size, poly(A)$_S$, thus stabilizing an mRNA population to be preserved for use in the egg after fertilization.

Localization and Function of Poly(A)⁺ RNA

The period of accumulation of poly(A)$_S$ coincides with the period during which the oocyte synthesizes the majority of its ribosomal RNA, and during which there is extensive replication of mitochondria. We have demonstrated that poly(A)$_S$ is not associated with ribosomal RNA, but its small size suggests that poly(A)$_S$ might be mitochondrial in origin. Quantitative considerations render this unlikely, since poly(A)$_S$ accounts for up to 70% of the poly(A)⁺ RNA of the oocyte, whereas only 0.5% of the ribosomal RNA (Chase and Dawid, 1972) and not more than 6% of the poly(A) (our unpublished data) of the full-grown oocyte are mitochondrial. That poly(A)$_S$⁺ RNA is transcribed from the nuclear genome is also more likely in view of the coding similarities between the two classes of poly(A)⁺ RNA, and it is also noteworthy that the accumulation of poly(A)$_S$ coincides with the lampbrush chromosome phase in oogenesis (Callan, 1963; Dumont, 1972).

In the full-grown oocyte the majority of the oocyte's poly(A)⁺ RNA is in the form of mRNP particles and is presumed to represent a store of mRNA which awaits utilization after fertilization (Rosbash and Ford,

1974). However, in the previtellogenic oocyte, in which poly(A)$_L$ is accumulating exclusively, 70% of the poly(A) is associated with polysomes (Darnbrough and Ford, 1976). During the later stages when poly(A)$_S$ is accumulating, the proportion of mRNP-associated poly(A) increases up to more than 90% in the full-grown oocyte (our unpublished observations). It remains to be seen whether the two classes of poly(A) represent populations of mRNA which differ in utilization or localization in the oocyte itself, or which are perhaps handled in different ways in the early embryo.

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